THE SCANDINAVIAN JOURNAL OF *Clinical & Laboratory Investigation*

EDITED FOR
THE SCANDINAVIAN SOCIETY
FOR CLINICAL CHEMISTRY
AND CLINICAL PHYSIOLOGY

Board of Editors

P. ASTRUP
H. O. BANG
E. BIERRING
E. BLEGEN
K. BRØCHNER-MORTENSEN
L. EFSKIND
L. ELDJARN
R. FRISK
N. HALLMAN
O. HELVE
C. G. HOLMBERG
B. JOSEPHSON
F.-E. KRUSIUS
J. LEHMANN
K. LUNDBÆK
P. A. OWREN
H. PALMER
A. PEKKARINEN
L. RAURAMO
F. SCHØNHEYDER
T. SJÖSTRAND
G. STRÖM
S. L. SVEINSSON
G. TÖTTERMAN

VOL. 13 æ 1961 æ NO. 2

PUBLISHED BY
MEDISINSK FYSIOLOGISK FORENINGS FORLAG
OSLO

THE SCANDINAVIAN JOURNAL OF CLINICAL AND LABORATORY INVESTIGATION

Managing Editor: Svein L. Sveinsson, M. D.
Associate Editor: Einar Blegen, M. D.
Editorial Secretary: Liv Theodorsen, M. D.
Editorial Office: Central Laboratory, Ullevål Hospital, Oslo, Norway.

EDITORIAL BOARD

Editors for Denmark: *Poul Astrup*, M. D., Head of Central Laboratory, Rigshospitalet, Copenhagen
Fritz Schønheyder, M. D., Professor of Biochemistry, University of Aarhus, Aarhus.
Editors for Finland (Suomi): *Aimo Pekkarinen*, M. D., Professor of Pharmacology, University o Turku, Turku.
Franz-Eduard Krusius, M. D., Docent, Head of Central Laboratory, Kivelä Hospital, Helsinki.
Editors for Norway: *Paul A. Owren*, M. D. Professor of Medicine, Head of Medical Dept. A, Riks hospitalet, Oslo.
Svein L. Sveinsson, M. D. Docent, Head of Central Laboratory, Ullevål Hospital, Oslo.
Editors for Sweden: *Rune Frisk*, M.D., Docent, Head of Medical Department, Ersta Sjukhus, Stockholm
Bertil Josephson, M. D., Docent, Head of Central Laboratory, S:t Eriks Sjukhus, Stockholm.
Section on Technique and Practical Problems: *Herbert Palmer*, M.D., Head of Central Laboratory Drammen Hospital, Drammen, Norway.

H. O. Bang, M. D., Head of Central Laboratory, City Hospital, Aalborg, Denmark.
E. Bierring, M. D., Head of Medicinsk Laboratorium, Vognmagergade 7, Copenhagen, Denmark.
Einar Blegen, M. D., Professor of Medicine, Head of Medical Department VII, Ullevål Hospital, Oslo, Norway.
K. Brøchner-Mortensen, M. D., Professor of Medicine, Head of Medical Department A, Rigshospitalet, University of Copenhagen, Denmark.
Leif Efskind, M. D., Professor of Surgery, Head of Surgical Department A, Rikshospitalet, Oslo, Norway.
Lorentz Eldjarn, M. D., Professor, Head of Central Laboratory, Rikshospitalet, Oslo, Norway.
Niilo Hallman, M. D., Professor of Pediatrics, Stenbäckinkatu 11, Helsinki, Finland.
Osmo Helve, M. D., Docent, Georgsgatan 2, Helsinki, Finland.
C. G. Holmberg, M. D., Professor, Head of Centra Laboratory for Clinical Chemistry, Lasarette Lund, Sweden.
Jörgen Lehmann, M. D., Professor, Head of Centra Laboratory, Sahlgrenska Sjukhuset, Gothen burg, Sweden.
K. Lundbæk, M. D., Professor of Medicine, Hea of Medical Department M, City Hospital, Aar hus, Denmark.
Lauri Rauramo, M. D., Docent, Olavinpuisto 2 B 18 Turku, Finland.
Torgny Sjöstrand, M. D., Docent, Head of Centra Laboratory of Clinical Physiology, Karolinsk Sjukhuset, Stockholm, Sweden.
Gunnar Ström, M. D., Professor, Head of Centra Laboratory of Clinical Physiology, Universit Hospital, Uppsala, Sweden.
Guido Tötterman, M. D., Professor of Medicine Head of Medical Department, Maria Hospita Helsinki, Finland.

The Scandinavian Journal of Clinical and Laboratory Investigation is edited for the Scandinavia Society for Clinical Chemistry and Clinical Physiology, and will appear quarterly, 4 issue forming a volume. Subscription price D. Kr. 45.00 + porto per volume in the Scandinavian countries. Othe countries D. Kr. 70.00. This price includes supplements.

The Scandinavian Journal of Clinical and Laboratory Investigation publishes original articles closel associated with clinical research and based on laboratory investigations. Pure methodological work of im portance for clinical research will also be accepted for publication. Authors in the Scandinavian countrie should send their manuscripts to one of the editors in their respective countries. Their addresses ar printed above.

Concerning subscription apply to *E. Munksgaard, Nørregade 6, Copenhagen, Denmark.*

For advertising apply to *Jacob C. Lund, M. D., Med. kjem. laboratorium, Sentralsykehuset, Trond heim, Norway.*

The four factors **in reverse** in the diagram are reduced by oral anticoagulant treatment. The combined activity of these factors is measured by Thrombotest.

INTRINSIC SYSTEM

Hageman factor
PTA (Anthihaemophilic C factor)
Factor IX (Antihaem. B factor)
Factor VIII (Antihaemophilic A factor)
Platelet factor 3
Calcium (Factor IV)

Factor X (Stuart-Prower factor)
Factor V (Proaccelerin)

EXTRINSIC SYSTEM

Tissue Thromboplastin (Factor III)
Factor VII (Proconvertin)
Calcium (Factor IV)

Factor X (Stuart-Prower factor)
Factor V (Proaccelerin)

Blood Prothrombinase

Tissue Prothrombinase

Prothrombin (Factor II)

Thrombin

Fibrinogen (Factor I)

Fibrin

MAXIMUM SIMPLICITY AND SENSITIVITY

An important advance in the control of anticoagulant therapy

THROMBOTEST

Information, scientific documentation and supplies available through:

G. Delforge & Co., Bruxelles
Erco Läkemedel, Stockholm
Ercopharm A/S, København
Evans Medical Ltd., Liverpool
Laboratoires Delagrange, Paris
Laboratori Biochimici FISM, Milano
Charles E. Frosst & Co., Montreal
OY Medica AB, Helsinki
N.V. Pharmachemie, Haarlem
Pharmacolor AG, Basel

Includes control of intrinsic as well as extrinsic coagulation system. Reagent is stable for one year. A standard correlation curve is provided with all packs. Reproducible results from one laboratory to another. Whole blood (**capillary** or venous) is used.

No expensive equipment needed.
After initial stabilization, control required only once a month.

NYEGAARD & CO. A/S - OSLO - NORWAY

1. 251. 1. 61

automation finally brought to clinical chemistry

Already in hospitals and laboratories the world over, the Technicon Autoanalyzer is doing jobs like those listed below with an accuracy, reproducibility, and dependability, which has users standing back in awe. **The work goes faster,** up to 60 tests-per-hour, **better,** accuracy is repeatable to approximately 1%, **and cheaper,** completely automatic; no human intervention or supervision.

There's a brochure which explains the Autoanalyzer principle, detailing operation and methods.

clinical biochemical analyses

For the thousand-and-one substances for which biological fluids are analyzed . . . blood glucose, blood urea nitrogen, calcium, alkaline phosphatase, chlorides, etc. Reproducibility, approximately 1%. Elapsed time-per-test, as little as one minute.

pharmacodynamic

Sampling by direct aspiration from circulatory (or other) system of a living organ, with continuous analysis and recording of phenomena like rate absorption or excretion of drugs, changes in concentration levels of blood urea nitrogen, blood glucose, etc.

completely automatic operation

Only human participation is initial setting up of test. Autoanalyzer then takes over; introduces correct proportions of materials and reagents, passes them through the train of analytical events — mixing, dialyzing, separation, heating, color-measuring, etc. — records test results as logarithmic or easy-reading linear graphs. Selectable choice of 20, 40, or 60 separate tests-per-hour, depending on nature of test. Reproducibility, approximately 1%.

TECHNICON®

AutoAnalyzer

Trade mark

TECHNICON INSTRUMENTS CORPORATION
CHAUNCEY, NEW YORK

Exclusive Representative for Sweden: **INGENIÖRSFIRMA HUGO TILLQUIST**
Wahrendorffsgatan 8, Stockholm 7, Tel.: 24 07 90 • Kullegatan 8, Gotëborg, Tel.: 20 92 90, 20 86 10

176,000 x g

at the touch of a button

MSE 'SUPER-SPEED 40' CENTRIFUGE

- 40,000 r.p.m. maximum speed
- Fully automatic electronically controlled operation
- Unique safety features include foolproof "overspeed" control
- Built-in vacuum and refrigeration units
- Extensive range of angle and swing-out rotors

REPRESENTATIVES:

DENMARK	Buch & Holm A.S., København K. Sølvgade 34—36
FINLAND	Kemistien Oy, Fabianinkatu 27 B, Helsinki
NORWAY	C. Grindvold, Tidemandsgt. 16, Oslo
SWEDEN	Axel Kistner, Lästmakargatan 22—24, Stockholm. Also at Göteborg and Malmö

MSE

MEASURING & SCIENTIFIC EQUIPMENT LTD., SPENSER ST., LONDON, S. W. 1. ENGLAND

INFRACORD

Många kliniska laboratorier och institutioner för medicinsk och klinisk kemi har redan anskaffat vår mindre registrerande infrarödspektrofotometer INFRACORD för såväl rutinanalyser som forskningsarbeten.

INFRACORD har ett våglängdsområde 2—15 μ och en upplösning, som är helt tillfredsställande för de allra flesta arbeten.

INFRACORD har tillbehör och tillsatser för undersökning av fasta — flytande och gasformiga prover.

INFRACORD har en synnerligen god mångsidighet genom ett stort antal tillbehör och tillsatser, såsom:

GASKYVETTER upp till 1 m
SPECIALKYVETT för uppfångning av fraktioner från gaskromatograf
UPPVÄRMBARA KYVETTER för accelererade lagningsförsök
POLARISATIONSTILLSATS
REFLEKTIONSTILLSATS
UTRUSTNING för KBr-teknik
ULTRA-MIKRO-TILLSATS för flytande prover
ULTRA-MIKRO-TILLSATS för KBr-teknik
GENOMSTRÖMNINGSKYVETTER

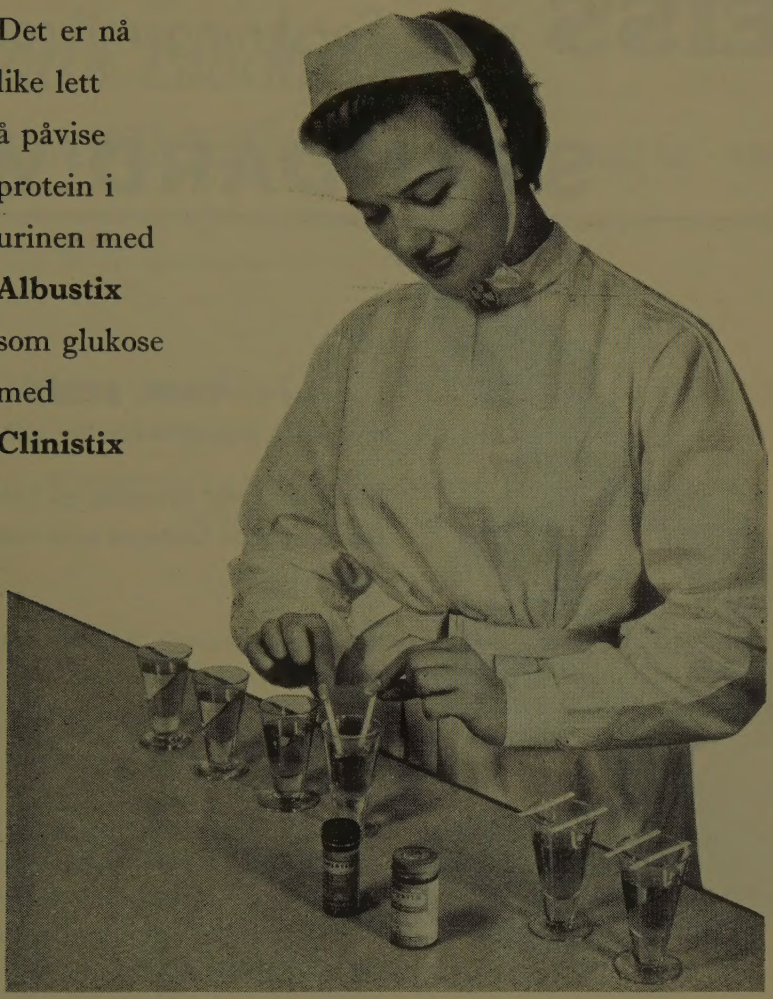
En utförlig 20-sidig broschyr väntar på Er.

FINLAND
Oy Control
Eerikinkatu 24
Helsinki
Tel. 13 505

NORGE
Nerliens Kemisk-Tekniske A/S
Tollbodgaten 32
Oslo
Tel. 41 73 43

DANMARK - SVERIGE
Perkin-Elmer
Fack 46020
Göteborg 46
Tel. 20 22 47 - 20 22 48

Det er nå
like lett
å påvise
protein i
urinen med
Albustix
som glukose
med
Clinistix



ALBUSTIX — Dip this end →

CLINISTIX — Dip this end →

gir begge resultat samtidig

Fåes på apotek, i drogeforretninger
og medisinske varehus

AMES COMPANY (London) Ltd. LONDON

ENEIMPORTØR: IVAR SCHJETLEIN — P. B. 1612 — OSLO

ZEISS *nye* Forskningsmikroskop

STANDARD WL

Det store nye **CARL ZEISS** mikroskop
med alle muligheder for udbygning.

Specialprospekt tilsendes på forlangende.
Demonstration foretages uden forbindende.

CARL
ZEISS

Vi står til Deres tjeneste:

ZEISS SVENSKA AB
Kungsgatan 33, Stockholm C
Telf. 24 02 00

BROCK & MICHELSEN
Vestergade 23, København K
Telf. 56 31

LORENTZ E. GJERSØE A/S
Kongensgate 2, Oslo
Telf. 42 54 16

THE
SCANDINAVIAN JOURNAL
OF
Clinical & Laboratory
Investigation

Vol. 13 · 1961 · No. 2

Scandinav. J. Clin. & Lab. Investigation 167—173, 13, 1961.

MEASUREMENT OF THE ELASTICITY AND COMPLIANCE OF THE LUNGS

DESCRIPTION OF A METHOD AND PRESENTATION OF SOME CLINICAL OBSERVATIONS

By T. K. I. LARMI[1] and R. APPELQVIST

From the Department of Thoracic Surgery, University of Helsinki, and from the Department of Physiology, Institute of Occupational Health, Helsinki, Finland

(Received for publication August 28, 1960)

The chest physician daily comes into contact with phenomena associated with the elastic properties of the lungs. The lung collapses when the chest wall is penetrated or the lung ruptures, and the difference in pressure between the external air and the pleural cavity decreases. At the same time as the lung contracts as a result of its elastic properties, the chest wall has a tendency to expand slightly, *i. e.*, to stretch outward. In various disease conditions, changes may occur in the elastic properties of the lungs or the chest wall or in the intrathoracic pressures, resulting in a disturbance of the respiratory mechanism and its reflection in the clinical picture of the disease.

Already in 1916 Rohrer presented his charts on the respiratory economy based on theoretic calculations. Thirty years passed before it was possible to demonstrate experimentally the interrelationship between the lungs, chest wall and the intrathoracic pressure (Rahn, Otis, Chadwick & Fenn 1946). Recently Donleben (1959) presented the sources of error in many currently used methods and their corrections. The methods

[1] Present adress: Mäntytie 10, Helsinki, Finland.

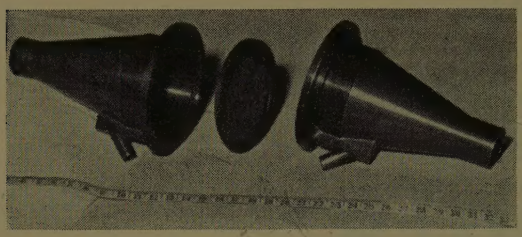


Fig. 1. *Flow meter* disassembled into its component parts, which are fastened together with screws to form an airtight unit. Note metal net (wires, 25 μ diameter, 400/in.) in the center.

in use have the drawback that they are either complicated or expensive. The object of the present paper is to present a simple and comparatively inexpensive method used by the author in the measurement of *compliance and elastance* of the lungs. Our experience with this method indicates in our opinion that it is well suited for clinical use.

DESCRIPTION OF THE METHOD

Apparatus. The *flow meter* used in the present investigation (Fig. 1) is similar to that described by Lilly (1950).

The *differential manometers* for determination of the pressure differences (a. on the two sides of a small-meshed metal net in the flow meter; b. intraesophageal/mouth pressure difference) were of identical construction. One of the gas pressures is led into the aneroid. A movable reflector is attached to the aneroid by means of an adjustable lever. The aneroid and its lever and reflector are enclosed in an airtight compartment of plastic sheets made airtight with rubber packing and screws so as to leave a minimum of air space between the aneroid and the plastic sheets. The other gas pressure is conducted into the space outside the aneroid. The outer plastic sheet has an airtight glass window over the reflector (Fig. 2).

The *source of light* was a lamp (6 V, 20—5 W), to which the current was connected through a transformer. The light was conducted through a metal tube with a narrow slit to a prism, whi distributed a linear ray of light onto the reflect in each of the two differential manometers.

The *photographic camera* was KIPP type S into which the line of light was directed vertica from the reflectors in the differential manomete When the aperture of the camera is a horizon slit, both vertical rays of light are recorded as d on the film turning in the camera. Two curves a thus recorded on the film simultaneously (Fig. One of the curves depicts the *rate of flow* and t other the *intraesophageal/mouth differential pr sure.*

The *intraesophageal pressure,* which is not e actly the same as the intrapleural pressure althou it lies close to it (*vide Donleben*), was measured the esophageal catheterization technique (F Stead, Ebert, Lubin & Wells 1952). A very thi latex balloon with a length of 13 cm and a volu of about 6 ml and attached to plastic tubing 2 mm internal diameter was introduced through t nose of the examined subject and into the e phagus to the level of the heart. The mouth pr sure was measured through a side tube attached the side of the mouthpiece (Fig. 3).

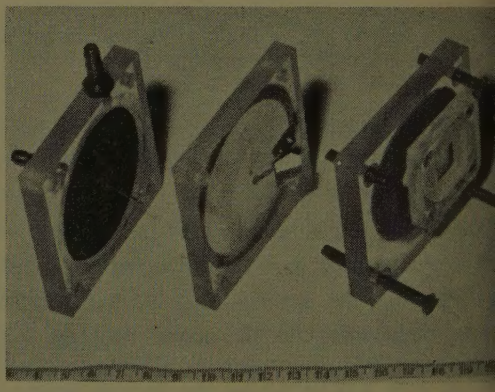
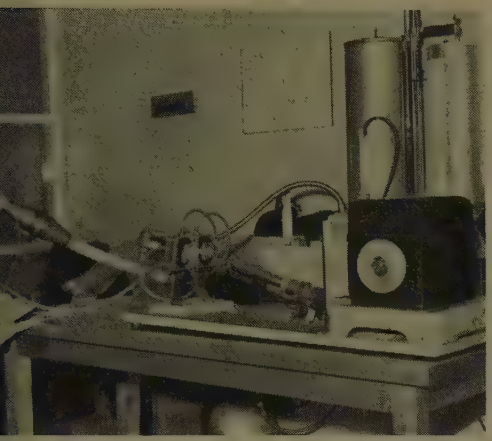


Fig. 2. *Differential manometer* consisting of aneroid and a reflector attached to the aneroid means of a lever. The manometer is enclosed a tight between plastic sheets fastened with scre One of the gas pressures to be differentiated conducted into the small air space between aneroid and plastic sheets. Outer plastic sheet a glass window over the reflector.



. 3. *Assembled apparatus.* Mouthpiece (on left) h attached side tube for measurement of mouth ssure, connected to the flow meter. Two plastic es lead from the flow meter to one of the differ- ial manometers. Another plastic tube, serving as phageal catheter, and the mouth pressure tube n the mouthpiece are connected to the other ferential manometer. Camera is seen on the right. rce of light and prisms are seen between the camera and the differential manometers.

The differential manometers, the light source h transformed, and the camera were attached to tand (Fig. 3). During measurement the entire nd was covered with a thin metal hood to pre- t light entering the camera (Fig. 4).

Procedures. The esophageal catheter was oduced through the subject's nose and the nose closed with a clamp. The mouthpiece was n inserted. This was a heavy plastic tube which connected to the flow meter (Fig. 3—4). The ject was *at rest in a comfortable sitting position.* was instructed to breathe calmly but was wed to choose freely his respiratory rate and l volume. The *tidal volume* was calculated rimetrically from the flow curve.

Calibration. Fig. 6 shows the calibration of flow curve. It will be observed that values up 30 liters/min. (2170 ml/sec.) can be calculated, ch is adequate for studies made at rest. Fig. 7 ws the calibration of the differential manometer esophageal/mouth pressures. The manometer gives a different calibration for low rates of flow than for high rates, and allowance has been made for this in the calculation. At fast rates of flow, pressure differences over ± 10 cmH_2O are not reliable.

Calculation. *Elastance of the lungs* is the ratio of the change in pressure to the change in lung volume. In other words, it indicates the change in the esophageal/mouth differential pressure when the lungs expand (or contract) by 1 liter. Therefore a greater pressure change is necessary to effect a given expansion of the lungs in "stiff" lungs than in "not stiff" lungs.

Compliance of the lungs is the inverse value of the preceding, and it states the change in liters in the lung "volume" produced by a pressure change of 1 cm H_2O.

Modulus of elasticity is the real indicator of the "stiffness" of the lungs (cm H_2O). It was calculated from the formula $E = SV.$, where $S =$ elastance of the lungs, and $V. =$ "initial volume" of the lungs (*i. e.,* functional residual capacity — T., where T. represents the "tidal volume" expired between the end-expiration level and the point when the intrapleural pressure is zero). V. is intermediary in size between residual volume and functional residual capacity; in ephysema it is the same as FRC (Donleben).

Fig. 4. Performance of measurement. During measurement the differential manometers and the source of light are covered with a light-tight metal hood. After esophageal catheterization the patient, in a sitting position, breathes through the mouthpiece into the flow meter. Cf. Fig. 3.

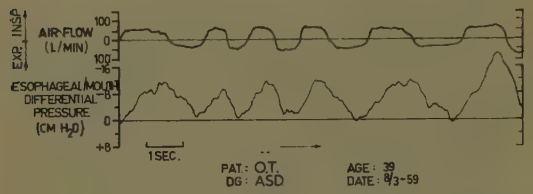


Fig. 5. Simultaneous air flow and esophageal/mouth differential pressure curves for male patient aged 40 (diagnosis: interatrial septal defect). Patient breathed at epproximately the same rate of flow but altered the tidal volume and respiratory rate.

Mean inspiratory resistance was calculated from the ratio between the initial and final pressure difference and the rate of flow. It states the pressure change occurring during inspiration when the rate of flow is 1 liter per second.

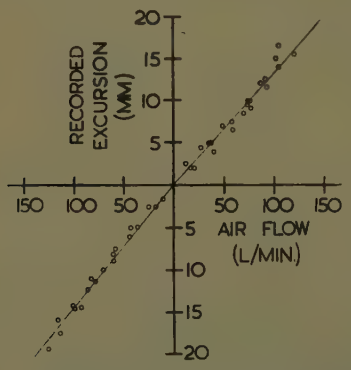


Fig. 6. Calibration curve of the flow meter.

RESULTS

Some Clinical Observations

The measurements in four healthy male subjects are given for comparison with the clinical series (Table I). The values correspond to normal values reported in the literature (McIlroy & Bates 1956, Donleben 1959, McIlroy & Marshall 1959).

From the data for 10 illustrative clinical cases it can be seen that primary disease of the lungs and primary heart disease cau stiffness of the lung tissue in a differe manner (Table II). In unilateral *cyst disease of the lung* some of the elasticity the lung is lost and the value for elastan is low and that for compliance relative high. *Atelectasis* of one lobe (case J. K., d to compression of bronchus caused by mali nant mediastinal lymphogranulomatosis) pr duced considerable stiffness. *Fibrosis of t lung* in bronchiectasis greatly reduced t expansion of the lungs and caused increas inspiratory resistance.

In *heart disease,* especially *mitral stenos* there are marked changes due to stiffne produced by an increased pulmonary arteri pressure (Larmi & Appelqvist 1960), a possibly also by secondary sclerosis and pr liferation of interstitial tissue (Larmi 1959

Four patients subjected to *lobectomy pneumonectomy,* were measured three wee after operations (Table III). The preoper tive values of two of these patients were i cluded in Table II. Postoperatively the ela tance of all these patients was above norm the compliance below normal, and the me

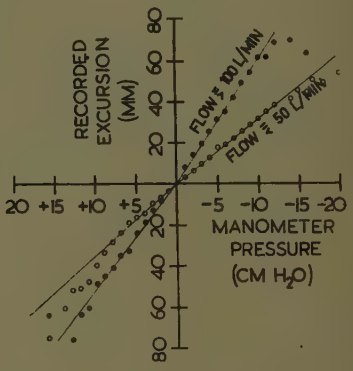


Fig. 7. Calibration curve of the esophageal/mo differential manometer.

Table I. *Physical Measurements of Some Visco-elastic Properties of the Lungs in Four Healthy Subjects.*

ur Healthy Subjects (Males)	Elastance cmH_2O/liter	Compliance liter/cmH_2O	Modulus of Elasticity cmH_2O	Respiratory Rate per minute	Rate of Flow ml/sec.	Mean Inspirator Resistance cmH_2O/liter/sec.
Mean	4.64	0.220	9.45	24	1695	4.47
Range	3.70–5.50	0.180–0.270	7.6–11.2	21.5–28.0	1610–2140	1.86–5.22
s* ±	1.175	0.0331	2.08	2.4	280	1.22

*s = standard deviation of the mean.

spiratory resistance elevated. It may be adily deduced (Fig. 8) that after pneuonectomy a greater esophageal/mouth differential pressure is needed to produce the me rate of flow within a given time at proximately the same respiratory rate and ith approximately the same tidal volume as fore operation.

DISCUSSION

The present method is suitable for clinical measurement at rest of the visco-elastic properties of the lungs. There is wide clinical application of the method. Christie (1953) stated that *dyspnea* and *orthopnea* in heart disease are a result of increased respiratory effort due to pulmonary congestion. The

Table II. *Physical Measurements of Some Visco-elastic Properties of the Lungs in Some Illustrative Clinical Cases.*

ubject, Age and Sex	Diagnosis	Elastance cmH_2O/liter	Compliance liter/cmH_2O	Respiratory Rate per minute	Rate of Flow ml/sec.	Mean Inspiratory Resistance cmH_2O/liter/sec.
.K., 37, M	Pulmo cystica unilateralis	4.0	0.248	22	1010	5.0
I., 50, M	Bronchial carcinoma	7.0	0.144	25	1225	7.4
K., 65, M	Atelectasis of middle lobe	7.8	0.128	19.5	895	10.2
.B., 20, M	Bronchiectasy	9.9	0.101	17	775	13.7
.T., 37, M	Bronchiectasy	10.6	0.094	23	535	18.7
.Tö., 40, M	Interatrial septal defect	5.1	0.200	23	850	5.8
.K., 21, M	Coarctation of the aorta	7.9	0.127	22	685	9.4
V., 25, F	Patent ductus arteriosus	8.9	0.112	25	715	11.9
.S., 37, F	Mitral stenosis	12.6	0.079	21	440	18.7
J., 28, F	Mitral stenosis	18.5	0.054	16	535	29.5

Table III. *Physical Measurements of Some Visco-elastic Properties of the Lungs in Four Subjects After Gross Resection of the Lungs.*

Subject, Age and Sex	Elastance	Compliance	Modulus of Elasticity	Respiratory Rate per minute	Rate of Flow	Mean Inspiratory Resistance
	cmH_2O/liter	liter/cmH_2O	cmH_2O		ml/sec.	cmH_2O/liter/sec.
A.K., 37, M**	10.3	0.098	28.1	22	1070	7.9
O.B., 20, M*	13.0	0.077		22	950	16.7
V.B., 60, M**	11.1	0.090		23	800	11.9
V.L., 67, M**	16.4	0.061		26	1550	13.3

*after lobectomy. **after pneumonectomy.

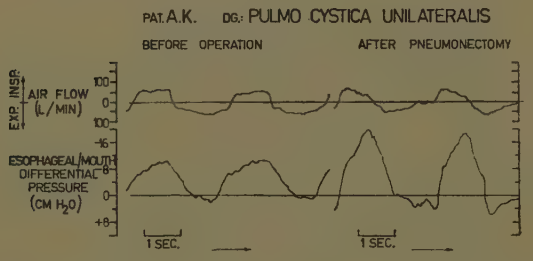


Fig. 8. Air flow curve and esophageal/mouth differential pressure curve determined *before and after pneumonectomy* in male patient aged 37 (diagnosis: unilateral cystic disease of lung). Rate of flow and respiratory rate were the same before and after operation, tidal volume was postoperatively slightly lower than preoperatively, but fluctuations in the esophageal/mouth differential pressure following pneumonectomy were twice as great as before operation.

patient chooses a respiration that causes a minimum of respiratory effort, *i. e.*, he generally increases the respiratory rate at the expense of the tidal volume. On the other hand, especially in emphysema, the non-elastic resistance of the lungs is increased. In voluntary hyperventilation the respiratory work is then greatly increased, whereas in hyperventilation caused by exercise it is decreased, contrary to that in heart disease.

Following *pneumonectomy,* one lung mus take care of the total ventilation. With eac inspiration the remaining lung must d "twice" as much work as normally. Its re serves therefore are only one-half of norma As a result, dyspnea develops more readil It is comprehensible that the elevated pres sure on the pneumonectomized side of th pleural cavity hampers the respiratory move ments of the remaining lung. For this rea son the patients are greatly relieved b equalization of the excess pressure. On th other hand, the excess pressure would pre vent overinflation of the remaining lun Reduction of compliance to less than one half of normal is considered a sign of ove inflation in the remaining lung (McIlroy Bates 1956).

The increased elasticity of the lungs fo lowing lobectomy in the present series ma also have been a result of elevated intra pleural pressure in the early postoperativ phase. Although lung function tests thre weeks after operation already show fair good results (Scheinin 1958), the visco elastic properties of the lungs and the che wall have evidently not as yet become equa ized and require a longer time for recover

SUMMARY

A method of measurement of lung elastic- and compliance using flow meter and phageal catheterization techniques is pre- ted. The mechanical differential mano- ters described are simple in construction l low in price. The method is suited for ical use for measurements at rest.

The authors report normal values and ical data obtained by this method con- ning the visco-elastic properties of the gs in certain lung and heart patients and four patients following pneumonectomy or ectomy.

REFERENCES

istie, R. V.: Dyspnoea in relation to the visco- elastic properties of the lungs. Proc. Roy. Soc. Med. *46,* 381, 1953.

leben, P. G.: Studies on lungmechanics. Thesis, Groningen, Drukkerij Romijn, Aperdoorn 1959.

, D. L., Stead, W. W., Ebert, R. V., Lubin, R. I. & Wells, H. S.: The measurement of intraesophageal pressure and its relationship to intrathoracic pressure. J. Lab. & Clin. Med. *40,* 664, 1952.

Larmi, T. K. I.: Pulmonary diffusion in some cardiovascular diseases before and after thoracic surgery. Ann. chir. et gynaec. Fenniae *48,* 518, 1959.

— & Appelqvist, R.: The influence of cardiac surgery on the mechanical properties of the lungs. Scandinav. J. Clin. & Lab. Investigation *13,* 174, 1961.

Lilly, J. C.: Flow meter for recording respiratory flow of human subjects, in Comroe, J. H. Jr.: Methods in medical research, vol. 2, Year Book Publishers, Inc., Chicago 1950.

McIlroy, M. B. & Bates, D. V.: Respiratory function after pneumonectomy. Thorax *11,* 303, 1956.

— & Marshall, R.: The mechanical properties of the lungs in asthma. Clin. Sc. *15,* 345, 1959.

Rahn, H., Otis, A. B., Chadwick, L. E. & Fenn, W. O.: The pressure-volume diagram of the thorax and lungs. Am. J. Physiol. *146,* 161, 1946.

Rohrer, F.: Der Zusammenhang der Atemkräfte und ihre Abhängigkeit vom Dehnungszustand der Atmungsorganen. Pflüger's Arch. f. d. ges. Physiol. *165,* 421, 1916.

Scheinin, T. M.: Studies of postoperative lung volume and lung function. Ann. chir. et gynaec. Fenniae *47,* Suppl. 78, 1958.

Scandinav. J. Clin. & Lab. Investigation 174—179, 13, 1961.

THE INFLUENCE OF CARDIAC SURGERY ON THE MECHANICAL PROPERTIES OF THE LUNGS

By T. K. I. LARMI and R. APPELQVIST

From the Department of Thoracic Surgery, University of Helsinki, and from the Department of Physiology Institute of Occupational Health, Helsinki, Finland

(Received for publication August 28, 1960)

As a result of heart surgery, some patients observe at a fairly early postoperative stage that their breathing is easier, whereas the relief of subjective symptoms in others requires a longer time even following a successful operation. The lung is readily distensible postoperatively by suction in some cases, but in other patients this may be difficult. Reasons for these differences may be the accumulation of, for example, blood and fluid into the pleural cavity, postoperative pain, and so on, but a difinite reason cannot always be found without special examination. Could a difference in the visco-elastic properties of the lungs possibly be the reason for this?

In respiration the organism must, during each inspiration, overcome a resistance that is equivalent to the sum of the elastic and non-elastic resistance of the lungs and chest wall, and the resistance of the turbulence and viscosity of air flow. In pathological conditions the increase in these resistances produces a feeling that "breathing is difficult". In certain heart diseases, such as mitral stenosis, the elasticity of the lungs and chest wall is reduced and respiratory work therefore is increased (Brown, Fry & Ebert 1954; Christie 1953). Are the mechanical properties of the lungs alter postoperatively? Theoretically this is po sible, since in mitral stenosis, for examp there occur changes in the pulmonary ar rial pressure, pulmonary blood volume, (Bing, Heimbecker & Falholt 1951; Gross Brockhoff & Schoedel 1958). The object the present paper is to present the exp rience we have had concerning visco-elas properties of the lungs in certain cardiac a vascular diseases before and after he surgery.

MATERIAL AND METHODS

Measurements were made on nine patients w mitral stenosis, four of whom were also stud postoperatively, eight patients with patent duc arteriosus, seven of whom also had a postoperat examination, and one patient with concretio cor and one with coarctation of the aorta, both of wh were studied pre- and postoperatively. The me urements were made 1—2 days before and 3 we after operation.

The mechanical properties of the lungs w measured with a *flow meter* (Lilly 1950) and the *esophageal catheterization technique* (Fry, Ste Ebert, Lubin & Wells 1952). The apparatus, p cedure, calculations and normal values have b described in another connection (Larmi & Ap qvist, 1961).

RESULTS

only two of the nine patients with *al stenosis* were the *elastance* and *com-ıce* within normal limits before opera-; in all the other patients the elastance increased and the compliance decreased. *modulus of elasticity* of the lungs and *mean inspiratory resistance* were in-sed in all the cases. The mean preoper-e values of the group are shown in le I. It was found that the higher the ıonary arterial pressure, the greater also elastance of the lungs (Fig. 1) and the ılus of elasticity. If the diastolic pul-ary arterial pressure was above 20 mm the elastance exceeded the normal limits. ostoperatively the values of three patients ained unchanged or reverted to normal, those of one patient were "poorer" in the respect that the average of the postoperative values showed a slightly increased "stiffness" of the lungs (Table II). The last mentioned patient was a woman aged 50 who

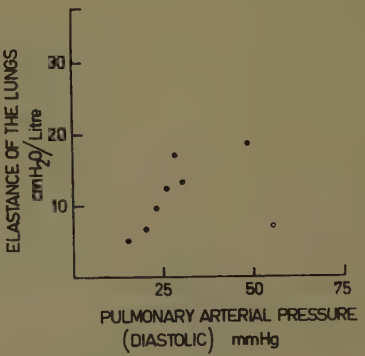


Fig. 1. Correlation of elastance of the lungs and pulmonary arterial pressure in eight cases with mitral stenosis. ● = cases without pulmonary emphysema; o = a case with pulmonary emphysema.

Table I. *Physical Measurements of Visco-elastic Properties of the Lungs in Mitral Stenosis and Patent Ductus Arteriosus Before Operation.*

Mitral Stenosis emale cases, ge range –50 years)	Elastance	Compliance	Modulus of Elasticity	Respiratory Rate per	Inspiratory Rate of Flow	Mean Inspirator Resistance
	cm H_2O/liter	liter/cm H_2O	cm H_2O	minute	ml/sec.	cm H_2O/liter/sec.
ean	11.0	0.117	21.8	22	880	12.1
nge	3.6–18.5	0.054–0.282	7.7–40.8	15.5–31	440–1560	4.4–29.5
±	4.16	0.0436	6.51	2.1	190	3.59
nt Ductus riosus male cases, range 0 years)						
ean	10.4	0.098	18.4	21	765	12.5
nge	8.9–14.2	0.070–0.112	12.7–27.2	16–29	535–895	9.3–21.3
±	3.08	0.0138	5.45	1.5	170	3.62
mal (4 healthy ects)						
nge	3.7–5.5	0.180–0.270	7.6–11.2	21.5–28	1610–2140	1.9–5.2

Table II. *Alteration of Visco-elastic Properties of the Lungs in Mitral Stenosis and Patent Ductus Arteriosus Three Weeks after the Operation (in Relation to Preoperative Values).*

Mitral Stenosis (4 female cases, age range 24–50 years)	Elastance cm H_20/liter	Compliance liter/cm H_20	Modulus of Elasticity cm H_20	Respiratory Rate per minute	Inspiratory Rate of Flow ml/sec.	Mean Inspirat Resistance cm H_20/liter/
Mean	+ 1.25	— 0.018	+ 2.3	— 1.0	+ 100	+ 3.28
Patent Ductus Arteriosus (7 female cases, age range 15–40 years)						
Mean	+ 4.6	— 0.025	+ 7.0	+ 3.0	— 14	+ 4.44

postoperatively developed pulmonary embolism, from which she later recovered.

In the *patent ductus arteriosus* group the preoperative values for elastance, modulus of elasticity and mean inspiratory resistance were above normal and compliance below normal in all the eight cases (Table I). This group did not reveal as marked variations as the mitral stenosis group.

Postoperatively most of these patients gave values indicating increased "stiffness", contrary to the finding in the cases of mitral stenosis (Table II).

The values for the patients with *coarc tion of the aorta* and *concretio cordis* shown in Table III. In the first mentio case, especially the modulus of elasticity high, althoug it fell slightly postoperativ From Fig. 2 it is seen that the inspirat rate of flow was the same but the e phageal/mouth differential pressure so what higher than preporatively, consequen the mean inspiratory resistance also is so what greather than preoperatively. The tient with concretio cordis, who preopera ely had severe dyspnea and pleural effus

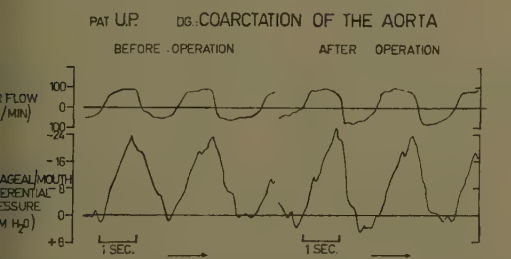
Table III. *Physical Measurements of Some Visco-elastic Properties of the Lungs in Coarctation of the Aorta (U.P.) and Concretio Cordis (V.R.) Before and Three Weeks After the Operation.*

Subject, Age and Sex	Elastance cm H_20/liter	Compliance liter/cm H_20	Modulus of Elasticity cm H_20	Respiratory Rate per minute	Inspiratory Rate of Flow ml/sec.	Mean Inspir Resistanc cm H_20/liter/
U.P., 20, M						
Before Operation	6.7	0.149	26.9	29.5	1310	7.0
After Operation	8.6	0.116	23.2	25	1310	10.5
V.R., 54, M						
Before Operation	9.7	0.103	18.9	20.5	1550	9.2
After Operation	6.0	0.167	12.3	29	1965	5.7

ddition to pulmonary stasis, had preoper-
e values indicative of considerable "stiff-
s" of the lungs, but the values were
atly normalized postoperatively. Fig. 3
ws this patient's flow curves and the eso-
geal/mouth differential pressure curves,
ich are rather exceptional. In the flow
ve there is no plateau during either in-
ration or expiration and the esophageal/
uth differential pressure undergoes an
upt change, with the exception of the
ldle of expiration, when there is practic-
no change.

DISCUSSION

n patients with mitral stenosis and patent
tus arteriosus in the present series the
in the preoperative elastance of the
gs, modulus of elasticity and mean in-
ratory resistance in comparison to normal
ues conforms with previous observations
rown *et al.* 1954; Mack, Grassmann &
tz 1947; Marshall & DuBois 1956). It
ears probable that this increased "stiff-
s" of the lungs in heart disease is related
elevated pulmonary arterial and capillary
ssures. In another study (Larmi & Ap-



2. Simultaneous recording of air flow and eso-
geal/mouth differential pressure in patient
coarctation of the aorta before and three weeks
after reparation of aorta isthmus stenosis.

pelqvist 1961) we were unable to find increased rigidity of the lungs in interatrial septal defect; however, an increase in pulmonary arterial or capillary pressures is generally not seen in these cases (Lagerlöff & Werkö 1949). In only one case of mitral stenosis in the present series was the pulmonary arterial pressure higher than would have been expected on the basis of the elastance. This patient, aged 50, also has emphysema, which either decrease elastance or increases compliance (Marshall & DuBois 1956; McIlroy & Marshall 1956).

In the case of coarctation of the aorta in this series and in case published by us in another connection (Larmi & Appelqvist 1961) the elastance of the lungs was slightly above normal, whereas the modulus of elasticity was greatly increased. It seems probable that in coarctation of the aorta an increased bronchial arterial pressure may be responsible for the increased rigidity of the lungs. It is true that the bronchial arteries arise from the third intercostal arteries, which in the present case lie distal to the coarctation, but since the bronchial arteries receive branches also from the internal thoracic artery or directly from the arch of the aorta,

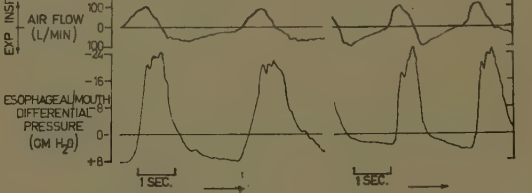


Fig. 3. Simultaneous recording of air flow and esophageal/mouth differential pressure in patient with concretio cordis before and three weeks after pericardectomy.

it is presumable that the increased pressure in these due to coarctation raises the pressure also in the bronchial arteries.

In concretio cordis, in which both the pulmonary arterial and (PAP) the capillary pressures (PCP) are somewhat elevated (Eliasch, Lagerlöf & Werkö 1950), an increased blood volume (PBV) (Bing *et al.* 1951) may be an additional cause of rigidity. Thus in the present case the elastance, modulus of elasticity, as well as the mean inspiratory resistance decreased postoperatively, possibly due to decreases in PAP, PCP and PBV, which have been observed following successful operation for concretio cordis.

In mitral stenosis the "stiffness" of the lungs is unchanged or decreased postoperatively, provided complications do not occur, as in one of the patients in the present series who developed pulmonary embolism. When studied at the corresponding time postoperatively, for example, the vital capacity in mitral stenosis was about 30 per cent below the preoperative values (Larmi, 1959). Thus the surgical operation undoubtedly has caused a favorable change in the pressures in the pulmonary blood circulation and pulmonary arteries and capillaries, resulting in diminished rigidity. Accordingly, changes in the visco-elastic properties of the lungs resulting from the operation probably also indicate the result of the operation. Pleural suction, important in postoperative treatment, should evidently be greater in those cases in which the postoperative pulmonary arterial pressure is higher in order to ensure equally good distention of the lungs.

It has been observed that in patients with mitral stenosis the respiratory work increases with increasing rigidity of the lungs, especially during exercise (Christie 1953). Therefore, if the increased "stiffness" of the lungs decreases postoperatively, diminished dyspnea may also be expected as a result of decreased respiratory work.

SUMMARY

The elastance, compliance, modulus of elasticity and mean inspiratory resistance of the lungs were measured by flow meter and esophageal catheterization technique in 17 cardiac patients before and in 13 patients also after surgical operation.

The "stiffness" of the lungs was increased preoperatively in seven of the nine patients with mitral stenosis, in the eight patients with patent ductus arteriousus, and in the one case of concretio cordis and one case coarctation of the aorta. Especially in the last mentioned case the modulus of elasticity was increased. In the mitral stenosis group the elasticity of the lungs was correlated with the pulmonary arterial and capillary pressures.

Postoperatively the values were unchanged or approached normal in three of the four patients with mitral stenosis studied, and in one patient the rigidity of the lungs increased, possibly due to pulmonary infarction after operation. In the patient with concretio cordis the "stiffness" of the lungs decreased postoperatively, in patent ductus arteriosus it decreased in two out of six patients and increased somewhat in the other four, and in the case of coarctation of the aorta the elastance and mean inspiratory resistance increased but the modulus of elasticity decreased.

REFERENCES

, R. J., Heimbecker, R. & Falholt, W.: An estimation of the residual volume of blood in the right ventricle of normal and diseased hearts *in vivo*. Am. Heart J. *42*, 483, 1951.

vn, C. C., Fry, D. L. & Ebert, R. V.: The mechanics of pulmonary ventilation with heart disease. Am. J. Med. *17*, 438, 1954.

stie, R. V.: Dyspnoea in relation to the viscoelastic properties of the lungs. Proc. Roy. Soc. Med. *46*, 381, 1953.

sch, H., Lagerlöf, H. & Werkö, L.: Diagnos av adhesiopericardit med särskild hänsyn till hjärtkatetrisering. Nord. med. *44*, 1128, 1950.

D. L., Stead, W. W., Ebert, R. V., Lubin, R. I. & Wells, H. S.: The measurement of intraesophageal pressure and its relationship to intrathoracic pressure. J. Lab. & Clin. Med. *40*, 664, 1952.

se-Brockhoff, F. & Schoedel, W.: Physiologie und Pathophysiologie des Kreislaufs, in Derra, E.: Handbuch der Thoraxchirurgie, Vol. I. Springer-Verlag, Berlin 1958.

rlöf, H. & Werkö, L.: Studies on the circulation of blood in man. VI. The pulmonary capillary venous pressure pulse in man. Scandinav. J. Clin. & Lab. Investigation *1*, 147, 1949.

Larmi, T. K. I.: Pulmonary diffusion in some cardiovascular diseases before and after thoracic surgery. Ann. chir. et gynaec. Fenniae *48*, 518, 1959.

Larmi, T. K. I. & Appelqvist, R.: Measurement of the elasticity and compliance of the lungs. Description of a method and presentation of some clinical observations. Scandinav. J. Clin. & Lab. Investigation *13*, 167, 1961.

Lilly, J. C.: Flow meter for recording respiratory flow of human subjects, in Comroe, J. H., Jr.: Methods in medical research. Vol. 2. Year Book Publishers, Inc., Chicago 1950.

Mack, I., Grassman, M. I. & Katz, L. N.: Pulmonary congestion and distensibility of lungs. Am. J. Physiol. *150*, 654, 1947.

Marshall, R. & DuBois, A. B.: Viscous resistance of lung tissue in patients with pulmonary disease. Clin. Sc. *15*, 473, 1956.

McIlroy, M. B. & Marshall, R.: The mechanical properties of the lungs in asthma. Clin. Sc. *15*, 345, 1956.

Scandinav. J. Clin. & Lab. Investigation 180—185, 13, 1961.

SOURCES OF ERROR IN THE DETERMINATION OF METABOLIC RATE

By J. GEORG and K. MELLEMGAARD

From Medical Dept. B, Rigshospitalet, Copenhagen, Denmark

(Received for publication September 1, 1960)

Some experience with a waterless bellows spirometer for measurement of basal metabolic rate have led us to a consideration of the sources of error in the determination of the oxygen consumption by closed circuit spirometry. The results of these considerations, together with some experimental evidence, form the substance of the present paper.

Stern & Strøm in 1957 published the results of a series of measurements of metabolic rate carried out with different apparatus. They found that measurements of oxygen consumption from collection of expired air (Douglas bag method) or from the volume changes of an ordinary closed system (Krogh spirometer) gave nearly identical values, the mean difference being —1.1 ml (standard deviation 15.4). With a bellows spirometer[1], however, they found the oxygen consumption on the average 17.8 ml/minute below the values determined by the Douglas bag method (standard deviation 15.1), a difference significant at the 0.1 per cent level.

Comparisons between determinations of oxygen consumption made with an open c cuit and with a water-sealed closed circ apparatus have been carried out seve times before, and no consistent differe between the two methods has been record (Carpenter 1915; Krogh & Rasmus 1922; Fowler, Blackburn & Helmholz 195 An explanation for the significantly lo values recorded with a bellows spirome was not offered by Stern and Strøm.

Similar findings were incidentally reco ed by Søttrup (1960) during a follow examination of patients convalescing fr poliomyelitis. He estimated the metab rate of 52 patients with a Krogh-spirome but at a second follow-up, two years la he used a waterless bellows spirometer. average metabolic rate was about 10 per c lower at the second follow-up examinati

We have made a similar comparison 10 subjects, all inpatients from a medical partment, and selected for the purpose account of their regular breathing produc spirograms which were specially suitable the measurement of oxygen consumpti The two types of apparatus were used random succession. The average oxy consumption measured with the Krogh sp

[1] Lundia Spirograf, model II (A.B. Instrumenta, Lund).

er was 237.2 ml/minute, with the bellows ometer 214.4 ml/minute, *i. e.* a difference .6 per cent in the oxygen consumption.

RESULTS

rces of error.

/hen the oxygen consumption is meas- l in a closed system the volume change he system is recorded during a certain . If the carbon dioxide produced by the ect is simultaneously absorbed, the vol- change represents the oxygen consump- , provided no other factors affect the vol- at the same time. Thus there are several

b l e I. *Sources of error in the deter- nination of basal metabolic rate by a closed circuit technique.*

age into or from the system:
Mouthpiece and valves
Nose clip
Perforated eardrum
Insufficient water in the spirometer
Leaking oxygen supply
rs of calibration:
Inaccurate volume scale
Faulty kymograph speed
ficient carbon dioxide absorption
ges in temperature during the test:
Heating of inspired air
Heat production of soda lime
Unfortunate placing of the apparatus (in sun- shine, near radiator)
ges in respiratory level of the subject:
Irregular respiration
Air-swallowing
Changes in position of subject
Changing pressure in the spirometer circuit
gen elimination from the body during oxygen thing
lus uptake of oxygen in subjects with low oxygen ration
ges in water vapor tension in the circuit

sources of error inherent in the method. They are listed in Table I. Leakage of the system is in general easily detected. The perforated eardrum is a rare source of error but has been noted among others by Switzer & Gates (1950). The calibration of the apparatus can be checked with little difficulty, and inefficient soda lime will manifest itself through hyperventilation of the subject.

Temperature changes.

Changes in temperature give rise to more serious errors, and for several reasons they are liable to influence the bellows spirometer more than the water-sealed spirometer. The first is a portable machine, which is usually carried to the patient, and thus more exposed to changing room temperatures. The water-sealed spirometer is preferably used as a stationary machine, being difficult to transport, and thus generally operated at a fairly constant room temperature.

Even with a quite constant room temperature there is still a tendency for the temperature of the apparatus to rise during the test. This is due to two factors: First, the heating of the inspired air, second, the heat generation in the soda lime during the neutralisation of carbon dioxide. Soda lime is a mixture of sodium and calcium hydroxyde. The neutralisation heat for these two bases is nearly the same, amounting to 10 kCal per gram-equivalent (Landolt-Börnstein 1923). The carbon dioxide elimination during an average test will thus produce 0.15—0.20 kCal per minute. The heat conveyed by the subject through the expired air matters less. The heat capacity of the air (20° C, 760 mm) is 0.24 cal/g, and assuming a 10 degree heating of the inspired air and a ven-

tilation of 5 litres per minute, this amounts to 0.015 kCal per minute or about 10 per cent of the total amount of calories added to the system.

In the water-sealed spirometer the conditions for heat dissipation are usually fairly good, the spirometer bell being of metal in direct contact with the surrounding air. The bellows of the dry spirometer do not convey the heat as easily, and besides they are often enclosed in a box.

With the bellows spirometer used in the present series of experiments we have recorded a consistent rise in temperature of 0.5 to 1° C (the temperature was measured by thermocouples placed in the circuit of the machine). The Krogh-spirometer under similar conditions showed no rise in temperature.

The changes in temperature are often greater during the actual tests in the wards. Measurements were made on the thermometer supplied with the bellows spirometer, and the following results were obtained in 100 different tests: Unchanged temperature: 24, one degree rise: 55, two degrees rise: 20, three degrees rise: 1. (All experiments where the initial temperature of the apparatus was below 18° C have been omitted.)

Heating of a certain volume of air under constant pressure leads to an expansion of 1/273 volume per degree C. The volume of the bellows spirometer used in these experiments is approximately 11 litres, and a rise of temperature of 1° C will correspond to an increase in volume of 40 ml, *i. e.* about 2 per cent of the oxygen consumption during a 10 minute test. This means that the oxygen consumption read from the kymograph record will be about 2 per cent too low.

Changes in water vapor tension.

The water vapor tension in a closed cuit will vary according to the type of sp meter. In an ordinary water-sealed sp meter the air will have an approxima constant water content, corresponding about 80 per cent saturation when soda l is included in the circuit (Wilson 192 The bellows spirometer, on the other ha

Table II. *The relative humidity of the in the metabolic apparatus before and after the test.*

Percentage water vapor saturation			
Krogh spirometer		*Bellows spirometer*	
before	after	before	after
80	76	34	52
88	87	32	81
82	80	38	68

starts with rather dry air and ends up w nearly saturated air. Table II shows degree of saturation with water vapor fore and after determination of basal me bolic rate with the two types of appara concerned. The measurements were done leading the air through magnesium perch rate, the amount of water absorbed be determined by weighing.

At a temperature of 22° C the tension saturated water vapor amounts to 20 Hg, *i. e.* a water content of 20/760 or 2.6 cent water vapor. A change in a spirome from completely dry to completely satura air will thus lead to an increase in volu of 2.6 per cent, or with a spirometer volu of 10 litres 260 ml, corresponding to m than 10 per cent of the oxygen consumpt during a 10 minute run. The changes water vapor saturation recorded in Table (from about 35 per cent to about 70 per c

uration) thus equal a volume change of ut 90 ml, and the oxygen consumption is respondingly recorded about 4 per cent low.

anges in the resting respiratory level.

A correct determination of oxygen con-nption from the spirometer curve is only sible when the respiratory level of the ject is kept fairly constant during the t. This is normally the case because the piratory position during quiet respiration resents a state of equilibrium, where the stic forces of the lungs (tending towards urther collapse) are equalled by the ela- forces of the thorax (tending towards a ther expansion). Any change in the press- in the airways will change this equili-um. The relation between volume and ssure change is expressed as the com-ance, which for thorax and lungs together a magnitude of about 100 ml per cm of ter pressure.

An ordinary water-sealed spirometer has alanced bell, causing the inside pressure ny position to be nearly atmospheric. The lows spirometer has a preferred resting ition, and any change in volume will lead a change in inside pressure. The relation pressure to volume for two bellows spiro-ters used in the present investigation is icted in Fig. 2. It will be seen that the ssure amounts to 8 mm of water with the lows expanded. During a determination metabolic rate, the volume decreases by ut 2—3 litres, and the pressure will fall proximately 5 mm of water. Assuming t the compliance for lung + thorax is ml per cm, this means that the respira-y level at the start of the experiment will

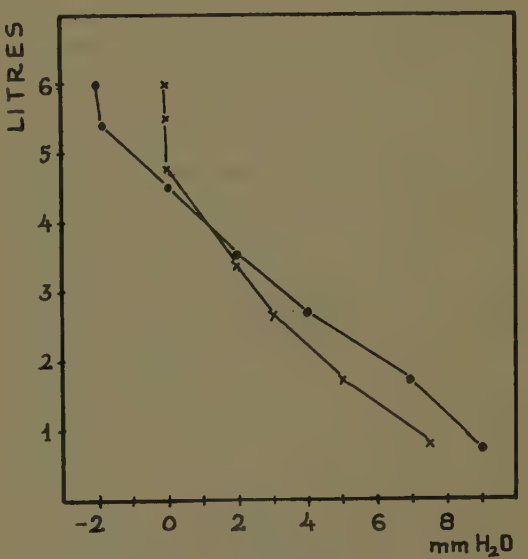


Fig. 1. Pressure — volume diagram of two bellows spirometers. Ordinate: Reading of spirometer scale. Abscissa: Pressure at the mouthpiece of the spirometer.

be shifted about 75 ml towards inspiration, but during the test about 50 ml will gradually be returned to the apparatus, and the slope of the spirogram will be correspondingly flattened. This means that the oxygen consumption will be estimated about 2 per cent too low. This source of error, inherent in the bellows type of metabolic apparatus, was first pointed out by Fleisch (1954), who was able to demonstrate the changes in respiratory level directly, using a body plethysmograph.

Elimination of nitrogen from the body.

The bodys store of nitrogen at the ordinary alveolar tension of about 565 mm is approximately 1 liter (Farhi & Rahn 1954—55). During respiration in oxygen, the nitrogen is gradually eliminated from the body, at the beginning rather quickly, after-

wards more slowly. The elimination curve represents a composite exponential function, the fastest process being the elimination from the lungs, then follows elimination from the blood, viscera, muscle tissue, fat, and eventually gas from the intestines (Jones, 1951).

The nitrogen elimination from the lungs is of no interest in the present study, as it does not alter the total volume of the system of spirometer plus lungs. The elimination from the body was estimated by Lundin (1953—54) at about 50 ml during the first minute, falling to about 15 ml during the tenth minute; the total amount eliminated during the first ten minutes being approximately 250 ml. Cournand, Yarmush & Riley (1941) got very similar results, the average being 220 ml for the first seven minutes of respiration in oxygen.

The experimental conditions are different during the determinations of oxygen consumption by closed circuit spirometry. Assuming a functional residual capacity of the subject of 3 litres, corresponding to 2.5 litres of nitrogen, and a spirometer volume of about 10 litres, the initial nitrogen concentration of the system will be about 20 per cent (provided the spirometer is filled with pure oxygen). During the test the nitrogen concentration will rise as oxygen is consumed, and may be expected to reach about 25 per cent at the end of a ten minute run. Correspondingly, the nitrogen elimination may be expected to be only about 70—75 per cent of the values cited above for respiration in pure oxygen, *i. e.* about 180 ml for the first ten minutes. This in turn leads to an estimation of metabolic rate 5—10 per cent too low. In actual practice the error will usually be much less, as the spirometer does not contain pure oxygen, but a mixture oxygen and atmospheric air. The error identical in principle for the two types metabolic measuring apparatus and th cannot explain the systematically low readings of the bellows spirometer.

If the apparatus is washed out with mospheric air before the test and has on 2—3 litres of oxygen added, approximate the amount consumed during the test, t error will be very small.

The increased oxygen concentration of t air in the spirometer may lead to a small i crease in oxygen uptake, especially if t arterial oxygen saturation is reduced. Th oxygen uptake will usually be complet during the preliminary test period of a fe minutes, before the actual measurement b gins. The increase in oxygen content of t body during the test is very small, as t venous oxygen tension is not very mu affected by a small increase in arterial ox gen saturation. Any errors from this sour will be more or less counteracted by t error in the opposite direction caused by t elimination of nitrogen, so for practical pu poses this source of error may be consider negligible.

CONCLUSION

There are several sources of error in t determination of oxygen consumption in closed system. Three of them seem espe ally liable to affect the readings of a wat less bellows spirometer, *viz.* rise in tempe ture, increase in water vapor tension, a variations in the respiratory level during t test, due to the slight pressure chan which accompany the altered position of t bellows.

ith the type of apparatus used in these riments, the three errors mentioned are oximately 2, 4 and 2 per cent of an ave- metabolic rate, all of them tending to results which are too low. Added her, they may be expected to produce ates about 8 per cent too low, a figure near the one observed during actual arisons between this and an ordinary r-sealed spirometer.

e errors are not difficult to reduce or nate. The increase in temperature may ractically abolished by means of a suit- placed ventilator, which keeps the soda canister at room temperature. The in- e in water-vapor tension may be counte- d by filling the spirometer with moi- d air instead of the ordinary dry air the oxygen cylinder. The pressure ges may be eliminated by suspending the ws with a counterpoise, keeping them in ant equilibrium. (Fleisch 1954.)

spirometer with these modifications at our disposal by *A. B. Instrumenta,* l) has given readings which are iden- with the values obtained on a Krogh meter. With 10 subjects the average en consumption was found to be 225.5 neasured with the Krogh spirometer, 5 ml measured with the modified water- bellows spirometer.

SUMMARY

e principal sources of error in the deter- tion of the oxygen consumption in a d system are discussed, with special ance to errors which may affect the ngs of different types of apparatus. It own that a waterless bellows spirometer to give lower readings than the water-sealed spirometer. Modifications for the elimination of these errors are proposed.

REFERENCES

Carpenter, T. M.: A comparison of methods for determining the respiratory exchange of man. Carnegie Inst. Publ. No. 216, Washington 1915.

Cournand, A., Yarmush, I. G. & Riley, R. L.: Influence of body size on gaseous nitrogen elimination during high oxygen breathing. Proc. Soc. Exper. Biol. & Med. *48,* 280, 1941.

Farhi, L. E. & Rahn, H.: Gas Stores of the body and the unsteadly state. J. appl. Physiol. *7,* 472, 1954—55.

Fleisch, A.: Nouvelles methodes d'etude des echanges gazeux et de la fonction pulmonaire. Basel, 1954, B. Schwabe.

Fowler, W. S., Blackburn, C. M. & Helmholz, H. F.: Determination of basal rate of oxygen consumption by open and closed circuit methods. J. Clin. Endocrinol. *17,* 786, 1957.

Jones, H. B.: Gas exchange and blood-tissue perfusion factors in various body tissue (in Fulton, J. F. (edit.): Decompression sickness. Philadelphia & London, 1951, W. B. Saunders).

Krogh, M. & Rasmussen, O.: Über Bestimmung des Energiumsatzes bei Patienten. Wien klin. Wchnschr. *35,* 803, 1922.

Landolt-Börnstein: Physikalisch-Chemische Tabellen Vol. II. Berlin 1923.

Lundin, G.: Nitrogen elimination during oxygen breathing. Acta physiol. Scandinav. Suppl. 111, 130, 1953—54.

Stern, B. & Ström, G.: Bestämning av syrgasupptagningen under basala betingelser. Nord. med. *57,* 243, 1957.

Switzer, J. L. & Gates, L. E.: The effect of a perforated eardrum upon the basal metabolic rate. J. Lab. & Clin. Med. *35,* 348, 1950.

Søttrup, T.: Studies on pulmonary function and diaphragmatic function in patients convalescing from life-threatening poliomyelitis. Copenhagen, 1960, E. Munksgaard.

Wilson, R. E.: Note on moisture absorbing efficiency of carbon dioxide absorbents for metabolism apparatus. Boston Med. & Surg. J. *187,* 133, 1922.

Scandinav. J. Clin. & Lab. Investigation 186—195, 13, 1961.

THE HELIUM DILUTION METHOD IN DETERMINATION OF VENTILATORY DISTURBANCES[1]

ITS APPLICATION TO BRONCHOSPIROMETRY

By I. STÅHLE

From The Pulmonary Function Laboratory, Kolmårdssanatoriet, Sweden

(Received for publication September 9, 1960)

Attention has frequently been drawn to the importance of bronchospirometric examinations for full elucidation of the function of each lung and, not least, for investigation of the pulmonary function prior to operations on the lungs. Results of residual volume determinations for each lung separately, both in normals and in turberculotics, have been reported from this hospital, and stress has been laid on the importance of such examinations in providing a comprehensive idea of the function of each lung, preoperatively and postoperatively (Orinius & Ståhle 1958). In this investigation the functional residual capacity (FRC) was determined by the helium dilution method.

Even in normal the ventilation of different parts in uneven to a varying extent. This has been established by studying the nitrogen elimination during inspiration of pure oxygen, using a rapid-response nitrogen analyzer (Fowler 1949, 1952). The borderline between this normally uneven alveolar ventilation and a pathologically uneven alve ventilation (PUV) is diffuse. Nor is it p sible, as a rule, to record a normally une ventilation by following the fall in hel concentration at determination of FRC the helium dilution method, the catharom response being too slow. It would seem, h ever, that a pathologically uneven ventila (PUV) can usually by recorded by follow this fall in concentration. Several diffe modes of treating the data obtained by helium dilution method have been repo with respect to studies of uneven alve ventilation.

If such a method of data analysis is t theoretically satisfactory, it should includ independent variables all factors that in ence the time required for indicator equilibrium between spirograph and lung a closed-circuit system (as in removal nitrogen from the lungs by an open-cir system technique). This equilibrium tim a function of respiration rate, tidal volu dead space, FRC and, in a closed-cir system, of the spirograph volume as These desiderata are satisfied only by

[1] The investigation was supported by a grant from Östergötlands läns landsting.

hod of Bates & Christie (1950), wherein calculated number of respirations is ted to the observed number of respira- s for 90 per cent equilibrium. Their hod, however, has been considered too rious for routine use.

rinius & Ståhle (1959) recently present- new method for data treatment of values ined at routine determination of FRC he helium dilution method in conjunction spirometry — a method which takes account the above requirements. The y of helium dilution in per cent of the ulated dilution time is the criterion ch, with this method, is determined as a sure of uneven alveolar ventilation. This ex (helium dilution delay or HDD) is puted with the aid of a nomogram which es it possible to establish rapidly whether ot clinically significant PUV is present. mals as well as a number of emphysema s have been investigated and the results ented, showing the reliability of this hod for data treatment.

here are numerous possible causes of V. One of the commonest is impaired elasticity, as for instance in emphysema. ther is regional bronchial obstructions, asthma, pulmonary cysts, and peri- and bronchial lesions. Tumors, pulmonary osis, and alveolar or interstitial fluid may onally alter the expansibility of the lung thus give rise to pathologically uneven olar ventilation. Yet another cause is an aired or impeded expansion of one or lungs, as in unilateral immobility of the hragm and/or the thoracic wall either wing pleurisy or lung surgery, or in nic nerve lesions (Comroe, Forster, Du- , Briscoe & Carlsen 1955). These diverse factors that may give rise to PUV seldom occur singly; two or more are usually concomitant.

In some cases it may be suspected clinically that *unilateral* PUV is present, as for example in shrinking processes of one lung with distension and impaired elasticity of underlying parenchyma, in unilateral pleural thickening following pleurisy or artificial pneumothorax, or in unilateral phrenic nerve lesions. At PUV determination in these cases, two possibilities are conceivable. This apparently unilateral PUV may be of such small extent in the diseased lung, or the latter's proportion of the total ventilation so slight, that spirometric determination of PUV will yield a normal value because the function of the normal lung predominates and masks the pathologically uneven alveolar ventilation of the other lung. The second possibility is that one lung has such pronounced PUV (in which case its relative FRC is fairly normal) that it prevails over the normal ventilation of the sound lung, the spirometric determination then showing a pathologic result. In this case the behavior may simulate bilateral PUV.

Apparently no attempts have yet been made to determine with a helium dilution method the degree of uneven alveolar ventilation in each lung separately at bronchospirometry. The aim of the present investigation is to ascertain if this method for data treatment (Orinius & Ståhle 1959) of values obtained during the course of helium dilution in spirometric FRC determination can also be used in bilateral bronchospirometric determinations of FRC for studying the occurrence of normally or pathologically uneven alveolar ventilation in each lung separately.

METHOD

Apparatus and Procedure.

The *bronchospirometer* was a double spirograph of Knipping type (Almara), modified to a volume, excluding the bellows, of 4.00 liters. The fan was adjusted to a capacity of about 50 liters per minute, and over it the catharometer was shunted. The circuit was so arranged as to include passage of the gas through a water bottle filled with glass beads for its saturation; and upon leaving the catharometer, the gas passed through a flow meter for ensuring a constant flow in the catharometer circuit. The temperature was continuously checked in both the spirograph and the catharometer circuit.

Spirometry was performed in the usual way in the supine position. Basal conditions were sought.

Bronchospirometry was carried out as follows. Premedication consisted of injection of 10 cg Phenemal and 1 mg atropine three quarters of an hour before the examination was begun. The anesthesia was administered in drops in all cases, 4 ml one per cent Novesin for the trachea. A Carlens tube was then introduced, size 37 for women and 39 or 41 for men. The subjects were supine and the examination began not earlier than about five minutes after the intubation. Basal conditions were sought.

Functional residual capacity (FRC) was determined by the helium dilution method as described by Holmgen (1954), with a few modifications. A small carbon dioxide filter, according to Bates & Christie (1950) was placed before the catharometer, and oxygen was added to the helium-air mixture *ad modum* Briscoe (1952).

The helium gas mixture was prepared by adding to the spirograph, flushed with air, four parts of helium and one part of oxygen to a helium concentration of about 13 per cent. The spirograph, including the bellows, then contained 5.2 liters. During subsequent rebreathing, an amount of oxygen equivalent to that consumed was supplied to the system, so that the respiratory curve had a horizontal course and the volume of the system was constant.

The apparatus was so arranged that the gas mixture was at room temperature when it reached the catharometer.

The examination opened with determination FRC, one lung being connected to the spirogra bell containing helium-air mixture and the oth lung to another bell containing oxygen. The reas for taking a concurrent spirogram of the other lu was to see that the resting expiratory level d not change as from the time when one lung w connected to the helium-air mixture and the oth to the oxygen spirograph.

When the helium concentration had remain constant for about two minutes in the examinati of the first lung, the "helium" lung was immediate connected to the oxygen spirograph. An interval about three minutes was allowed before the "ox gen" lung was connected to the helium spirogra

During rebreathing, each helium fall of 0.2 p cent was recorded on the spirograph paper ma ually with the aid of an electro-magnetic device.

Following the determination of FRC, the heli spirograph was flushed several times with oxyg then filled with oxygen. Both lungs were then co nected to the system, after which the ventilati and the oxygen uptake were measured for b lungs simultaneously over a four-minute period.

All volumes are given at ambient pressure a saturated with water vapor. FRC is corrected the volume of the mouthpiece and for possi errors relative to resting expiratory level wh connecting the patient to the spirograph (alwa less than 100 cc). No adjustment has been ma on the other hand, for the effect of oxygen on helium-air mixture or for the absorption of heli in blood and tissue.

For further details of apparatus and proced the reader is referred to Orinius & Ståhle (19 1959).

Method.

It was found that the optimal range of accur for the dilution time corresponded to 60—80 cent of completed dilution with this apparatus; in this range 63 per cent was taken as a compa tive level, since it was mathematically advantage to some degree.

The time required for 63 per cent dilution adjusted for the initial delay but not for the

is lag. Adjustment for the former is done by polation to

$$\frac{He_t - He_\infty}{He_0 - He_\infty} = 1. \qquad (I)$$

eoretically this extrapolation, if it is to be linearly must be undertaken in a logarithmic/ system, though it can be done, with very little error, within the line of markings on the gram.

e time required for the value to reach 63 per dilution of the indicator gas (helium) between graph and lung may be approximated.

$$t_{0.63\ calc.} = \frac{60}{\dot{V}_A \left(\frac{1}{V_L} + \frac{1}{S}\right)} \qquad (II)$$

e $\dot{V}_A$ is the alveolar ventilation in liters per e, and V_L = FRC and S the spirograph vol- all in liters. These calculations can be further ified by devising a nomogram (Fig. 1).

e average respiration rate and tidal volume determined from the spirogram for the re- t dilution time, *i. e.* the observed time for brium minus 9 seconds (equation III below) ounded off to the nearest multiple of 5 seconds. mputing the alveolar ventilation a dead space allowed for, which, from the bifurcation, was as- d to be 40 cc for each lung in males and 35 cc nales. Even if the dead space varied by 10 cc or minus those volumes, it would seldom in- a change of more than one second in the ated equilibrium time.

The observed dilution times, corrected for the l delay in the spirograph-catharometer-galva- ter system, were plotted against the times uted on the basis of equation (II) above. For er details see Orinius & Ståhle (1959).

RESULTS

nal series.

even males aged from 22 to 27 (mean 25) and eight females aged from 18 to mean age 23) were examined. One of had previously had uncomplicated bronchial pneumonia; none of the others had suffered from lung disease. Chest roentgenograms had been taken in all but two of them during the few months preceding the examination. The roentgenograms were negative in all subjects, none of whom showed clinical signs of respiratory tract disease. Data on the pulmonary function will be found in Table I.

There was a close correlation, in these normals, between the observed and the calculated dilution times, see Fig. 1. Both male and female subjects and the values for right and left lung showed a uniform distribution relative to the regression line. The regression line was found to be as follows.

$$t_{0.63\ obs.} = 1.03 \times t_{0.63\ calc.} + 9.2 \qquad (III)$$

as against

$$t_{0.63\ obs.} = 1.05 \times t_{0.63\ calc.} + 8.3 \qquad (IV)$$

the latter equation having been obtained in a spirometrically normal series (58 subjects) with the same technique and apparatus (Orinius *et al.* 1959).

The variation about the regression line was 1.8 seconds. If, as an upper limit for normal values, we take the mean + 2.5 × standard deviation, the equation for that upper limit will be

$$t_{0.63\ obs.} = 1.03 \times t_{0.63\ calc.} + 13.7 \qquad (V)$$

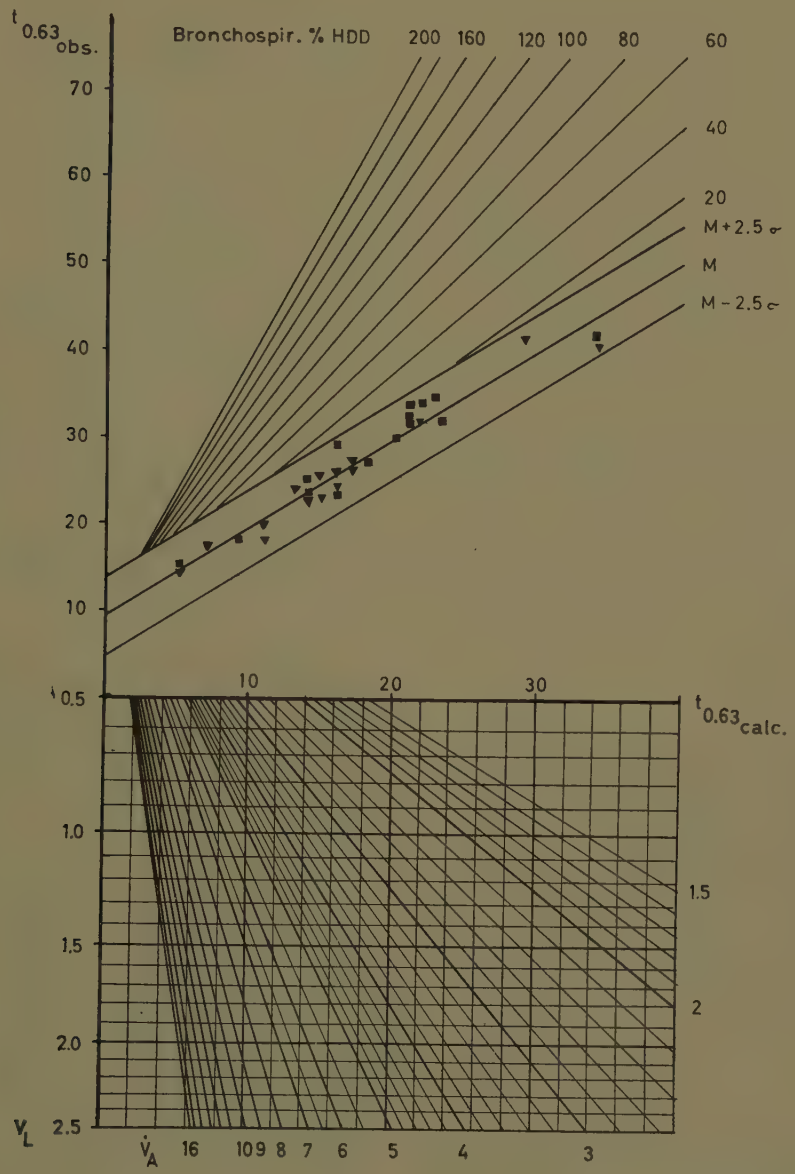
Discussion.

There is, accordingly, a close correlation between the observed and the calculated helium dilution times both in spirometric and in bronchospirometric determination of FRC by the helium dilution method. The standard deviation is, however, somewhat greater in bronchospirometric (1.8 seconds) than in spirometric (1.0 second) determina-

Table I.

Subject	Sex	Age years	Lenght cm	Weight kg	Vital Capacity		Functional Residual Capacity		Residual Capacity		Total Lung Capacity		FRC/TLC per cent		RV/TLC per cent		One Second Vital Capacity	Maximal Breathing Capacity	Helium Dilution Delay	
					S lit.	Br %	S lit.	Br %	S lit.	Br %	S lit.	Br %	S	Br	S	Br	per cent S	liter S	per cent S	Br
G. L.	M	25	190	81	7.0	L48 R52	3.8	L47 R53	2.3	L46 R54	9.3	L47 R53	41	L42 R43	25	L25 R27	82	180	0	L 0 R 0
P. K.	M	27	169	66	4.3	L46 R54	1.6	L41 R59	0.9	L46 R54	5.2	L46 R54	31	L27 R34	18	L14 R14	88	103	0	L 0 R 0
G. K.	M	27	180	73	5.6	L50 R50	2.7	L51 R49	1.5	L52 R48	7.1	L50 R50	38	L35 R35	21	L18 R16	88	147	0	L 0 R 0
J. G.	M	26	177	64	4.3	L48 R52	2.7	L46 R54	1.4	L45 R55	5.7	L48 R52	46	L51 R51	24	L27 R30	93	152	0	L 0 R 0
T. H.	M	22	174	65	4.7	L48 R52	2.3	L47 R53	1.1	L45 R55	5.8	L47 R53	40	L37 R37	19	L18 R19	81	124	0	L 0 R 0
R. W.	M	26	175	75	5.2	L48 R52	3.0	L50 R50	1.4	L49 R51	6.6	L48 R52	45	L45 R43	21	L22 R22	84	136	0	L 0 R 0
B. J.	M	27	172	82	6.3	L49 R51	2.5	L43 R57	1.5	L43 R57	7.8	L48 R52	32	L30 R36	20	L20 R24	82	119	0	L 0 R 0
B. N.	F	36	161	66	3.7	L48 R52	1.8	L47 R53	1.5	L48 R52	5.2	L48 R52	35	L38 R40	28	L27 R26	84	114	0	L 0 R 0
B. J.	F	20	161	57	3.3	L47 R53	1.6	L44 R56	1.0	L49 R51	4.3	L48 R52	38	L33 R38	22	L19 R18	90	89	0	L 0 R 0
M. J.	F	20	168	56	3.5	L51 R49	2.1	L50 R50	1.3	L50 R50	4.8	L50 R50	44	L44 R46	27	L28 R29	92	98	0	L 0 R 0
I. H.	F	18	160	53	2.9	L45 R55	1.8	L47 R53	1.2	L50 R50	4.1	L47 R53	43	L46 R46	30	L31 R27	91	76	0	L 0 R 0
T. P.	F	32	176	56	4.4	L49 R51	2.8	L47 R53	1.7	L47 R53	6.1	L48 R52	45	L45 R47	28	L29 R31	84	120	0	L 0 R 0
G. H.	F	22	178	65	3.8	L48 R52	2.2	L46 R54	1.4	L48 R52	5.2	L48 R52	42	L43 R46	28	L26 R26	86	80	0	L 0 R 0
G. B. J.	F	22	165	53	3.2	L46 R54	2.0	L47 R53	1.5	L46 R54	4.7	L46 R54	42	L41 R43	33	L27 R28	88	87	0	L 0 R 0
A. M. J.	F	18	169	60	4.2	L47 R53	1.8	L45 R55	1.0	L49 R51	5.2	L47 R53	34	L35 R38	20	L24 R23	95	96	0	L 0 R 0

RV = residual volume
FRC = functional residual capacity
TLC = total lung capacity
S = spirometry
Br = bronchospirometry
L = left



. *Upper half.* $t_{0.63\,obs.}$ in seconds, plotted t $t_{0.63\,calc.}$ in seconds for normal men (▼) omen (■). The regression line of the nor- ubjects (M) and its upper (M + 2.5) and (M —2.5) limits are marked. The lines 20 to 200 represent per cent of helium dilution delay (HDD) relatively calculated dilution time.

Lower half: nomogram for calculation of $t_{0.63\,calc.}$, based on a volume of the spirograph of 5.2 liters. V_L = FRC. V_A = alveolar ventilation in liters per minute.

tion. Two factors at least contribute to this circumstance.

(1) Constant methodologic errors which, by virtue of smaller lung volume subdivisions in bronchospirometry, have a greater influence on the final result; and

(2) The error introduced by approximation is somewhat increased, as shown by the expression for this error as a correction factor for the calculated dilution time

$$K = 1 + \frac{V_A}{2}\left(\frac{1}{V_L} - \frac{1}{S}\right) \qquad \text{(VI)}$$

where S is the spirograph volume — identical in spirometry and bronchospirometry while V_A (the alveolar ventilation per respiration) and V_L (FRC) are volumes which, though substantially smaller in bronchospirometry than in spirometry, show more or less similar ratios.

Pathologic Series.

From an extensive clinical series of pulmonary cases, investigated both spirometrically and bronchospirometrically with respect to helium dilution delay (HDD), the following seven with involvement chiefly of one lung were selected with a view to illustrating various possible results of HDD determination. Data on the pulmonary function are set forth in Table II.

Case Reports.

1. B 3188. Male, aged 22. In 1954 the patient had tuberculosis with widespread parenchymal lesions of the right lung. Right artificial pneumothorax was maintained from 1954 to 1957. Right progression occurred in 1957 and again in 1958. Roentgenograms showed cavitation of right upper lobe and right pleural thickening following artificial pneumothorax. Left lung N.A.D.

At spirometry, HDD was normal: at bronc spirometry it was normal for left but pathol (30 per cent) for right lung. FRC for right was 44 per cent of the total.

2. B 36 19. Male, aged 55. He has had asthm bronchitis since 1954; bronchoscopy and bronc graphy in autumn of 1959 revealed stenosis of r lower lobe bronchus and ectatic bronchi in lower lobe, where there was chronic pneumo Left lung N.A.D.

At spirometry, HDD was normal; at bronc spirometry it was normal for left but pathol (10 per cent) for right lung. FRC for the r lung was 43 per cent of the total.

3. B 3523. Female, aged 29. In 1953 she very slight parenchymal lesions posteriorly in right upper lobe. Right artificial pneumotho was maintained from 1953 to 1958, followed severe pleural thickening. Left lung N.A.D.

At spirometry HDD was normal; at bronc spirometry it was normal for left but pathol (40 per cent) for right lungt. FRC for right was 31 per cent of the total.

4. B 3256. Male, aged 30. In January widespread cavernous tuberculosis of left lung detected, and total destruction of lung ensued. very slight parenchymal lesions were observe the right lung field.

At spirometry, HDD was normal; at bronc spirometry it was pathologic (80 per cent) for but normal for right lung. FRC for left lung 19 per cent of total.

5. B 3159. Male, aged 49. Chronic nonspe pneumonia with bronchectasies in right lower leading in January 1956 to excision of that Phrenic nerve was probably traumatized at operation, the sequel being a high and practi immobile diaphragm on the right side. The lung field was free.

At spirometry in December 1958, HDD pathologic (50 per cent); at bronchospiromentr was normal for left and pathologic (150 per for right lung. FRC for right lung was 42 per of normal.

6. B 3379. Male, aged 34. Patient had left pl isy in 1942, followed by moderate pleural thicke In 1946 there were bilateral upper lobe les mainly on the left side. In the ensuing years

Table II.

Patient number	Sex	Age years	Lenght cm	Weight kg	Vital Capacity		Functional Residual Capacity		Residual Capacity		Total Lung Capacity		FRC/TLC per cent		RV/TLC per cent		One Second Vital Capacity per cent	Maximal Breathing Capacity per cent	Helium Dilution Delay per cent	
					S lit.	Br %	S lit.	Br %	S lit.	Br %	S lit.	Br %	S	Br	S	Br	S	S	S	Br
1. B 3188	M	22	176	75	3.6	L62 R38	1.9	L56 R44	1.4	L54 R46	5.0	L62 R38	38	L31 R36	28	L22 R27	64	55	0	L 0 R 30
2. B 3619	M	55	168	61	4.1	L59 R41	4.0	L56 R44	3.1	L63 R37	7.2	L55 R45	56	L53 R50	43	L35 R25	47	65	0	L 0 R 10
3. B 3253	F	29	169	61	3.1	L74 R26	1.7	L69 R31	1.2	L67 R33	4.3	L72 R28	40	L39 R45	28	L29 R36	85	67	0	L 0 R 40
4. B 3256	M	30	184	68	2.6	L19 R81	1.9	L20 R80	1.3	L19 R81	3.9	L19 R81	49	L57 R53	33	L43 R43	82	58	0	L 80 R 0
5. B 3159	M	49	174	78	2.9	L63 R37	2.5	L58 R42	2.1	L52 R48	5.0	L59 R41	50	L50 R53	42	L39 R53	64	35	50	L 0 R 150
6. B 3379	M	34	177	83	4.2	L39 R61	2.1	L43 R57	1.7	L40 R60	5.9	L39 R61	36	L30 R26	29	L20 R19	60	55	50	L 70 R 0
7. B3 510	M	37	176	62	1.9	L59 R41	3.2	L52 R48	2.8	L50 R50	4.7	L55 R45	68	L54 R62	60	L39 R48	44	27	90	L 80 R 140

RV = residual volume
FRC = functional residual capacity
TLC = total lung capacity

S = spirometry
Br = bronchospirometry
L = left
R = right

factory regression; but in 1959 progression and cavitation were observed in the left lung.

At spirometry, HDD was pathologic (50 per cent), at bronchospirometry it was pathologic (70 per cent) for left but normal for right lung. FRC for left lung was 43 per cent of normal.

7. B 3510. Male, aged 37. Bilateral pulmonary tuberculosis since 1951: right artificial pneumothrax from 1951 to 1955, left from 1953 to 1955. This treatment was followed by extensive bilateral pleural thickening. During the next few years cavernous tuberculosis developed in both upper lobes. Right thoracoplasty was done in July 1959.

Spirometry in November 1959 showed pathologic HDD (90 per cent); at bronchospirometry in same month, HDD was pathologic for both left (80 per cent) and right (140 per cent) lungs. FRC was 52 per cent for left and 48 per cent for right lung.

DISCUSSION

It was pointed out earlier that patients with pulmonary lesions solely or chiefly involving one lung may be expected to show pathologically uneven alveolar ventilation only in that lung. It was also emphasized that spirometry in such cases might well yield both normal and clearly pathologic values for the degree of uneven alveolar ventilation.

The first of the cases presented above had pronounced unilateral pulmonary tuberculosis with pleural thickening following artificial pneumothorax, but fairly normal relative FRC values for the two lungs. Case 2 had a stenosis of the right lower lober bronchus, with destruction of this lobe as well as ectatic areas there. Here too, the relative FRC was fairly normal for the diseased lung. These two cases illustrate the first of the aforementioned two possibilities; a normal result of spirometric determination of uneven alveolar ventilation despite unilateral PUV. In each of these cases the PUV of the diseased lung was so slight that, in spite of the r latively normal FRC percentage for th lung, the spirometric value for the uneve alveolar ventilation was within the norm range.

The patient in case 3 had a pronounce unilateral pleural thickening following art ficial pneumothorax, with minimal pare chymal lesions and a greatly reduced FR figure for the affected lung. In case 4 the was a pulmonary process involving one lu which had undergone a total destruction a showed a greatly reduced proportion of t total FRC. These two cases illustrate t second of the two possibilities; namely, th spirometry may show a normal value for t uneven alveolar ventilation despite marke PUV in one lung if the latter's proportio of the total FRC is low.

Case 5 had right phrenic nerve lesio following excision of the right lower lob The PUV was pronounced on that side; t FRC value in relation to the sound lung w fairly normal. In case 6 there were som what extensive lesions of the upper lobe a pleural thickening following pleurisy on t diseased side; the bronchospirometric PU was fairly marked and the relative FRC mo or less normal for that lung. These cas illustrate the possibility that one lung m have a normally uneven alveolar ventilati which is completely masked spirometrical partly because the diseased lung has PU of high degree and partly because, at t same time, its proportion of the total FRC practically normal.

The patient in case 7 had severe lesio of both upper lobes with cavitation a shrinkage, as well as bilateral pleural thic ening following artificial pneumothora

spirometric and bronchospirometric inations yielded, as was to be expected, values showing pronounced PUV netrically as well as in each lung ately..

SUMMARY

e helium dilution and its delay were ed for each lung separately in conjunc- vith determination of functional residual ity in normals and tuberculotics, using um dilution method and bronchospiro- . A nomogram for calculating uneven ar ventilation (helium dilution delay DD) in each lung separately is present- ; are the values recorded in a series of rmal subjects.

e results of such HDD determinations, netrically and bronchospirometrically, escribed in seven cases with largely uni- l parenchymal lesions, bronchostenosis, l thickening and phrenic nerve injury. s pointed out that a normal HDD value netrically is not tantamount to a nor- uneven alveolar ventilation in both ; pathologically uneven alveolar ven- n may well be present in the diseased but either concealed by that lung's y reduced relative FRC, or itself of extent and attended by a normal or vhat lowered relative FRC.

ilarly, a pathologic HDD at spiro- is not necessarily tantamount to bi- l pethologically uneven alveolar ven- n. On the contrary the normally uneven alveolar ventilation in the sound lung may be masked by a highly pathologic uneven alveolar ventilation in the diseased lung, whose relative FRC may then be normal or slightly reduced.

REFERENCES

Bates, D. V. & Christie, R. V.: Intrapulmonary mixing in health and in emphysema. Clin. Sc. *9*, 17, 1950.

Briscoe, W.: Further studies on the intrapulmonary mixing in normal and emphysematous subjects. Clin. Sc. *11*, 45, 1952.

Comroe, J. H., Forster, R. E., Dubois, A. B., Briscoe, W. A. & Carlsen, E.: The lung: clinical physiology and pulmonary function tests. The Year Book Publishers Inc., Chicago 1955.

Fowler, W. S.: Lung function studies. III. Uneven ventilation in normal subjects and patients with pulmonary disease. J. appl. Physiol. *2*, 283, 1949.

— Lung function studies VIII: Analysis of alveolar ventilation by pulmonary N_2 clearance curves. J. Clin. Investigation *31*, 40, 1952.

Holmgren, A.: Determination of the functional residual volume by means of the helium dilution method. Scandinav. J. Clin. & Lab. Investigation *6*, 131, 1954.

Orinius, E, & Ståhle, I.: Determination of bilateral residual volume. I. Examination of healthy subjects. Am. Rev. Tuberc. & Resp. Dis. *78*, 368, 1958.

— Determination of bilateral residual volume. II. Examination in cases of pulmonary tuberculosis. Am Rev. Tuberc. & Resp. Dis. *78*, 376, 1958.

— The helium-dilution method in determination of ventilatory disturbances. A new method for data treatment. Am. Rev. Tuberc. & Resp. Dis. *79*, 450, 1959.

Scandinav. J. Clin. & Lab. Investigation 196—204, 13, 1961.

SAMPLING AND STORING OF BLOOD FOR DETERMINATION OF ACID-BASE STATUS [1]

By O. SIGGAARD ANDERSEN

Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark.

(Received for publication September 13, 1960)

The purpose of the present paper is to discuss the possible errors occurring during sampling and storing of blood for determination of pH, pCO_2 and base excess (BE). The most likely errors during sampling are loss of CO_2 by exposure to atmospheric air, dilution in connexion with the addition of anticoagulant and effects of the anticoagulant itself. To these errors may be added the error involved in the addition of sodium fluoride when this is used as antiglycolytic agent. During storing the metabolism of the blood will change the acid-base values. The effect of chilling and of sodium fluoride in prevention of this change will be discussed.

METHODS

A "thermostated" capillary glass electrode was used for pH measurements. pCO_2 and base excess (meq/l blood) were calculated from the actual pH value and from the pH value of the blood equilibrated with a known CO_2-tension (Siggaard Andersen, Engel, Jørgensen & Astrup, 1960 and Siggaard Andersen & Engel, 1960).

Hemoglobin concentrations were determined spectrophotometrically as oxy-hemoglobin. So and potassium were determined by means direct reading flame-photometer (Beckman). cose was determined by reduction titration a ding to Hagedorn and Norman-Jensen.

The blood was kept in two syringes conn by a small rubber tube so that the blood cells be resuspended after storing of the blood by ing the blood from one syringe to the other.

Reagents:

Powdered heparin (LEO) was used for preparation of solutions with different conce tions of heparin.

Sodium fluoride (A. R.) and sodium chl (A. R.) was obtained from Merck.

RESULTS

A) *Loss of CO_2.*

By the previously described method sampling of capillary blood for determina of the acid-base status (Siggaard Ande *et al.* 1960), the blood is exposed for a time to atmosperic air. The effect of was investigated by emptying drops of b of the same size (approximately 50 µl) a syringe on to siliconated glass slides w the blood drops formed hemispheres. two minutes the pH of the blood was

[1] This work was supported by grants from "The Danish Foundation for the Advancement of Medical Science" and "The Danish State Research Foundation".

both by sucking up directly into the ode from the central part of the drop y letting the blood nearly quantitatively nto a capillary tube where it could be l before measurement of the pH. By rst method a mean pH rise of 0.005 found. By the second method a mean ise of 0.010 was found.

Dilution effect.

e effect of 12—13 per cent dilution of and plasma with physiological saline investigated. The following mean es were found in 4 experiments with a: pH rise 0.003, pCO_2 fall 14 per actual bicarbonate fall 13 per cent, base s fall 3.5 meq/l. The mean changes in periments with whole blood were: pH 0.006, pCO_2 fall 16 per cent, plasma bonate fall 15 per cent, base excess fall eq/l.

hen the blood is sampled in a syringe the dead space filled with a heparin on a certain dilution of the blood is able. In a series of ten 10 ml syringes en 20 ml syringes (Summit, Everett) ead space in percentage of the maximal ne of the syringes were found to be 2.4 per cent and 2.0—3.0 per cent ctively.

Heparin effect.

e effect of heparin on the acid-base was investigated by stabilizing the with 0.01 mg/ml and adding heparin concentrations of 2, 4 and 10 mg/ml. experiments a practically linear depend- of the heparin concentration was found. The calculated effect of a concentration of 1 mg/ml was: pH fall 0.003, pCO_2 rise 0.1 mm Hg and base excess fall 0.2 meq/l.

The effect in lipemic blood was specially investigated by comparing actual pH measured with and without the use of heparin (0.4 mg/ml). 4 experiments showed no significant difference.

D) *Acid-base changes in anaerobically stored blood.*

The changes in pH, pCO_2 and base excess were investigated in blood stored anaerobically at 38°, 20°—24° and 0°—4° C.

The initial pH changes (0—30 min.) were investigated by filling the electrode with blood, closing the open end of the electrode with a rubber band and following the pH reading. Capillary blood from 8 normal individuals was examined. The electrode was filled immediately after blood sampling both with heparinized blood (0.4 mg/ml) and with unheparinized blood. In all 8 cases a practically straight line pH fall was found both when the electrode was "thermostated" at 38° C and at room temperature. The rate was 0.066 ± 0.014 pH units per hour at 38° and 0.026 ± 0.006 at 22—24° C. No difference was found between heparinized and unheparinized blood. Storing of the blood for ½—1 hour at 38° C, room temperature or 0° C before measurement did not influence the course of the pH change at 38° C. Comparison of venous and capillary blood showed no difference.

The course of changes in pH, pCO_2 and base excess for longer times was investigated by frequent measurements on blood stored in a syringe. An example of the course of

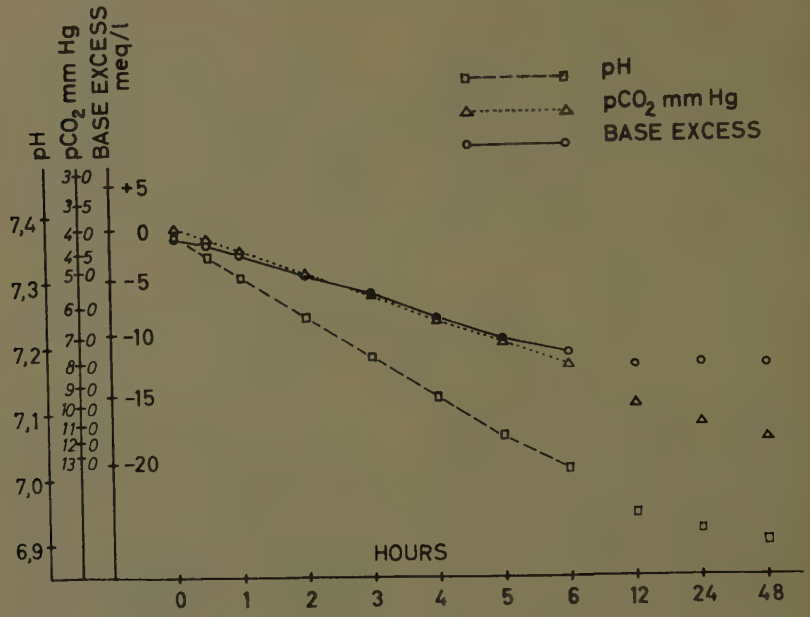


Fig. 1. Changes in the acid-base status of human blood stored anaerobically at 38° C. The diagram used is described by Siggaard Andersen 1960.

changes in normal blood at 38° C is shown in Fig. 1. It is seen that all three values change practically in a straight line for the first 3 hours at 38° C. Then all three tail off and reach in the course of 24 hours: pH 6.9, BE —10 to —15 meq/l and pCO_2 10 150 mm Hg. The rate of change for the t values in the first 3 hours at different tem rature is shown in the Table. As seen, changes within the first three hours

Table I. *Changes in acid-base status of human blood stored at different temperatures. The rate of changes per hour for the first 3 hours of storage is given as mean value and range of results.*

Temperature of storage	Number of experiments	△ pH per hour	△ BE meq per hour	△ pCO_2 mm Hg per hour
38° C	10	— 0.062 ± 0.021	— 1.9 ± 0.4	4.8 ± 1.3
22—24° C	5	— 0.024 ± 0.012	— 0.5 ± 0.2	2.5 ± 1.1
0—4° C	4	— 0.006 ± 0.004	— 0.1 ± 0.2	0.6 ± 0.6

imal when the blood is stored at -4° C.

n examination of the cause of the differ- in the rate of changes in different blood ples showed that the rate is independent he hemoglobin concentration but very endent on the number of leukocytes. In a ple of a suspension of leukocytes (6,400 ocytes per μl, hemoglobin concentration g/ml) the following changes were found: pH fall 0.067 per hour, BE fall 1.8 meq/l per hour and pCO_2 rise 5.3 mm Hg per hour.

E. *Effect of sodium fluoride on blood acid-base status.*

This effect can be separated into two parts with different causes: 1) The simple salt effect and 2) the effect on the changes in the acid-base status due to the enzyme inhibitory action of the fluoride ion.

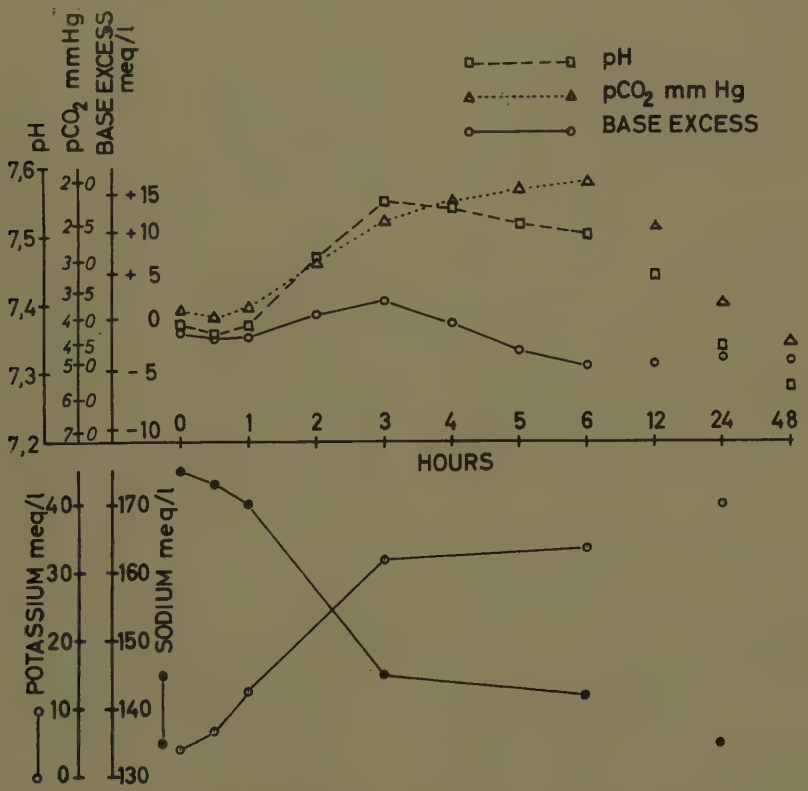


Fig. 2. Changes in the acid-base status of human blood (same as shown in Fig. 1) plus sodium fluoride to a concentration of 1 mg per ml blood and stored at 38 ° C. The changes in the sodium and potassium concentrations of the plasma are also shown.

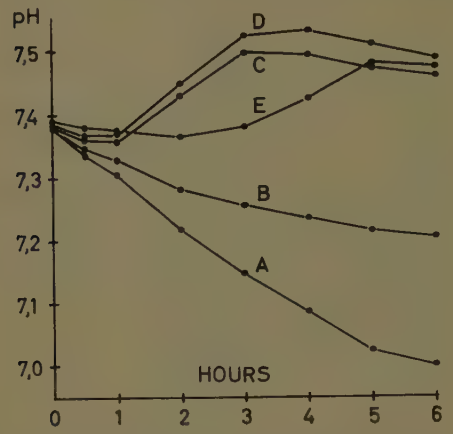


Fig. 3. The effect of different fluoride concentrations on the pH changes in human blood stored at 38° C. The amount of sodium fluoride in mg per ml blood was zero (curve A), 0.25 (curve B), 0.5 (curve C), 1.0 (curve D) and 2.0 (curve E.)

1) The simple salt effect was investigated in 14 experiments with a concentration of sodium fluoride in the blood of 5 mg per ml and the effect was compared with that of sodium chloride in equimolar concentration. In five experiments the powdered salts were added. In the rest of the experiments the salt was first dissolved in physiological NaCl which was then added to the blood in the ratio $^1/_{10}$ and comparison was made with blood diluted to the same ratio with pure physiological NaCl. The acid-base status was determined within 15 minutes after the addition of the salt. With different commercial preparations of sodium fluoride different results were obtained, but with pure sodium fluoride (A.R.) in freshly prepared solution reproducible results were obtained and the same effect was observed with equimolar concentrations of sodium chloride and sodium fluoride. At a concentration of 5 mg per ml the effects in whole blood were: pH ri 0.030, pCO_2 fall 6 mm Hg and BE fall 1 meq/l. The effects on plasma were: pH fa 0.030, pCO_2 fall 2 mm Hg and BE fall 2 meq/l. The use of different concentratio from 1 to 5 mg per ml showed approximate a straight line dependence. The effect 1 mg/ml whole blood can concequently anticipated as a pH rise of 0.006, a pC fall of 1.2 Hg and a BE fall of 0.3 meq/l.

2) The changes in acid-base status blood plus sodium fluoride to a concentrati of 1 mg per ml during storing at 38° C we investigated in 7 experiments all showi identical changes. One of the experiments illustrated in Fig. 2. The common chang in the 7 experiments were as follows: ½- hour after the addition of fluoride the p value reaches a minimum having fallen 0.0 —0.020. Simultaneously the BE falls 0.5— meq/l and pCO_2 rises 0—2 mm Hg. Aft this, the course turns to the basic side a after 3—4 hours the pH reaches a maximu after a rise of 0.17—0.19. Simultaneously, shortly before, the BE reaches a maximu after a rise of 2—3 meq/l. The pCO_2 reach a minimum somewhat later, after about 5— hours, having fallen approximately 20 m Hg. After this, the values again change t wards the acid side, and, after 24 hours, t starting point is almost reached, pH bei 0.020—0.060 lower, BE being 2—4 me lower and pCO_2 almost the same.

In six of the experiments the change acid-base status was also followed in t blood plus an equimolar amount of Na and in the blood without added salt. T course of changes could not be distinguish whether NaCl was added or not. Deran ment of the osmotic equilibrium due to

salt is consequently of minimal im-
ıce for the course of changes and the
of NaF must be considered to be
ic.

e effect of different concentrations of
of 0.25 mg, o.5 mg, 1 mg and 2 mg per
ood was examined and the effect on pH
wn in Fig. 3. It is seen that 0.25 mg/
uses an even change to the acid side
as the other concentrations show the
to the basic side. The major change
n with a concentration of 1 mg/ml, and
r concentrations apparently delay the
e.

e effect of 1 mg/ml of NaF was invest-
l at 22° C. The same course of changes
ınd at 38° C occurred, only somewhat
ed. The pH minimum (—0.021) oc-
l after 1—1½ hour and the maximum
7—8 hours.

determine the cause of these peculiar
es, the effect of 1 mg/ml of NaF on a
nsion of leukocytes in plasma (6,400
cytes per µl, 0.1 g hemoglobin per 100
nd on saponin hemolyzed blood kept at
was investigated. In both cases an
change to the acid side occurred in
ourse of ½—1 hour after which the
s were practically constant or very
y changing to the acid side. The initial
hange in the leukocyte suspension was:
all 0.050, BE fall 2 meq/l, pCO_2 rise
Hg, and in the hemolyzed blood: pH
.050, BE fall 0.5 meq/l and pCo_2 rise
Hg.

concentration of 1 mg NaF per ml blood
ound to prevent fall in glucose concen-
n effectively in blood stored 24 hours
° C. Apparently some acid-producing
cal processes other than glycolysis not inhibited by NaF are the cause of the initial change to the acid side in fluoridated blood. The change to the basic side appears to be dependent on the presence of intact red cells and must be due to a redistribution of acids or bases between the red cells and plasma. To elucidate this further the distribution of K and Na in fluoridated blood was investigated. The results are shown in Fig. 2. It is seen that the potassium rapidly leaks out of the cells in the course of 3 hours and that sodium in the same time leaves the plasma to enter the cells. In blood without fluoride the sodium and potassium concentrations were practically constant within 3 hours at 38° C. To investigate whether changes in hydrogen ions were connected with the simultaneous changes in K and Na, dog blood was examined because of the small difference in electrolyte concentrations between cells and plasma in dog blood. Here no basic shift was found, and only small changes in plasma potassium (4.2 to 6.3 meq/l) and plasma sodium concentrations (182 to 172 meq/l) occurred within 3 hours at 38° C.

DISCUSSION

A) *Loss of CO_2.*

In 1934 Shock & Hastings described a method for the sampling of capillary blood under liquid paraffin. In 1955 Singer, Sohl & Bluemle described a "virtually anaerobic" technique where the blood dripped into a small funnel from where it was immediately sucked into a small syringe. They compared the two methods and found greater Co_2 loss by sampling under paraffin than by the virtually anaerobic technique. The present in-

vestigation shows that the loss of CO_2 from a blood drop is a rather slow process without practical significance when the capillary blood is sampled by a virtually anaerobic technique as in the one described by Singer, Sohl & Bluemle or the one described by Siggaard Andersen *et al.* 1960.

B) *Dilution effect.*

The dilution effect on blood pH was described by Yoshimura (1935) who found no change in blood pH on 10 per cent dilution with physiological saline. The present data confirm the fact that blood pH is practically unaffected by small dilutions. The actual bicarbonate concentration of plasma and the pCO_2, however, fall in direct proportion to or slightly more than the dilution, and the base excess falls about 0.3 meq/l per per cent of dilution.

C) *Heparin effect.*

Several authors have shown that the effect of heparin on the acid-base status is minimal for a concentration of 1 mg per ml blood. (Yoshimura 1935; Severinghaus, Stupfel & Bradley 1956). However, Sanz (1960) has stressed the importance of avoiding heparin, as small concentrations may cause a considerable acid shift, especially in lipemic blood, possibly due to the co-lipase activity of heparin. This effect could not be demonstrated in the present investigation.

D) *Acid-base changes in anaerobically stored blood.*

The use of the capillary glass electrode for following the changes in the pH reading of the solution filling the electrode are discussed in the following paper by Siggaard Andersen. It was shown that with a pr electrode the spontaneous drift in the reading of a plasma sample stored in electrode at 38° C was 0.012 pH units hour. The spontaneous drift with wh blood must be considerably lower due to better buffering capacity of whole blood. attempts were made to correct the obser changes in whole blood pH for the sp taneous basic drift. The mean value for rate of pH change (0.066 ± 0.014 per ho for blood stored in the electrode agrees sely with that of blood stored in syrin (0.062 ± 0.021).

In 1928 Havard & Kerridge describe "first acid change" of 0.07 in the course 10—12 minutes after shedding of the blo Thereafter a much slower change occur due to glycolysis. The change could be p vented by rapid cooling but not by fluor The present investigation has not sho such a rapid first acid change. Both at ro temperature and at 38° C the pH fell appr imately as a straight line for several ho

It is shown that cooling to 0—4° C p vents changes of any practical significance the acid-base values for at least 3 hours. L of potassium from the red cells and ba shift apparently come considerably later. irreversible changes due to cooling fo hours were found.

E) *Effect of sodium fluoride on blood a base status.*

The simple salt effect of the NaF on actual pH value has been described by veral authors. Yoshimura (1935) found a concentration on 2 mg/ml lowered the value by 0.100. Møller (1959) found 2.5 mg/ml increased the pH value by 0.0

e present work showed that 5 mg/ml in-ased the pH value by 0.030. This di-epancy is possible due to the fact that mmercial NaF may contain considerable ounts of impurities. Data on the effect on O_2 and base excess could not be found in literature.

The most likely errors in the acid-base ues when a concentration of 1 mg NaF ml of blood is used and the blood is ed at 22—24° C for 0—3 hours before asurement are as follows: The immediate effect causes an increase in pH of 0.006 a decrease in pCO_2 of 1.2 mm Hg, and ecrease in BE of 0.3 meq/l. During stor-for 3 hours the maximum changes will pH fall 0.010—0.020, pCO_2 rise 0—2 Hg and BE fall 0.5—1 meq/l. The over-effect will be a pH error from +0.006 to ut —0.014, a pCO_2 error from —1 to +1 Hg and a BE error from —0.3 to —1.3 /l. In a previous publication (Siggaard lersen *et al.* 1960) 10 comparisons be-en arterial and capillary blood were orted. For actual pH no significant differ-e was found. For pCO_2, and base excess value in capillary blood was 2 mm and —1 meq/l lower respectively. Only the llary blood was stabilized with NaF mg/ml) whereas the arterial blood was pled in a syringe and subject to a dilution bout 2 per cent. It seems that the differ-e is simply explained by these errors.

1959, Nunn described the effect of ride on the course of changes in whole d pH, but he did not report the effect on O_2 or base excess. The present invest-on completely affirms Nunn's findings. an be concluded that NaF does not pre-an initial acid production from some other sources than glucose. The fluoride, however, prevents the glycolysis necessary for the maintenance of the uneven distribution of the cations between the cells and the plasma. The change to the basic side in fluoridated blood is obviously due to this effect of the fluoride but the true mechanism has not been elucidated. It is supposed that the transient change to the basic side is due to diffusion potentials arising as long as the K and Na distribution is out of equilibrium causing a change in the distribution of H^+. It can not be entirely excluded, however, that active processes maintain an uneven distribution of H^+ in human red cells by pumping H^+ out of the red cells like sodium, and that this mechanism is blocked by fluoride, so that H^+ enters the cells until equilibrium is attained. This mechanism, however, offers no explanation of the fact that the shift is transient.

CONCLUSIONS

Some directions for the sampling and storing of blood for determination of the acid-base status may be given on the basis of the present investigations.

Heparin may be used in concentrations below 1 mg per ml without significant error.

Corrections must be applied for an eventual dilution as pCO_2 falls percentwise and BE falls approximately 0.3 meq/l per 1 per cent dilution. The actual pH value is practically unchanged.

The blood should be stored for as short a time as possible and best chilled in icewater. Within 3 hours no significant changes in acid-base status occur at 0—4° C. If this is not practicable the blood can be stored for

about 3 hours at room temperature (22—24° C) when sodium fluorides in a concentration of 1 mg per ml blood is used. This, however, introduces a variable error of +0.006 to —0.014 for pH, —1 to +1 mm Hg for pCO_2 and —0.3 to —1.3 for base excess. (—0.2 to —1.0 meq/l for standard bicarbonate.)

SUMMARY

The effects on the blood acid-base status (pH, pCO_2 and base excess) of exposure of the blood to atmosperic air, of heparin, of dilution of the blood, of glycolysis and of sodium fluoride have been investigated. Some directions for the sampling and storing of blood for determination of the acid-base status are given.

REFERENCES

Havard, R. E. & Kerridge, Phyllis T.: An immediate acid change in shed blood. Biochem. J. *23,* 600, 1928.

Møller, Bent: The hydrogen ion concentration in arterial blood. A clinical study of patients with diabetes mellitus and diseases of the kidneys, lungs and heart. Universitetsforlaget i Aarhus 1959.

Nunn, J. F.: The accuracy of the measurement of blood pCO_2 by the interpolation techique. In Woolmer, R. F.: A symposium on pH and blood gas measurement. J. & A. Churchill, London 1959.

Sanz, M. C.: Personal communication. Geneva 1960.

Severinghaus, J. W., Stupfel, M. & Bradley, A. F.: Accuracy of blood pH and pCO_2 determinations. J. appl. Physiol. *9,* 189, 1956.

Shock, N. W., & Hastings, A. B.: Studies of the acid-base balance of the blood. I. A micro technique for the determination of the acid-base balance of the blood. J. Biol. Chem. *104,* 565, 1934.

Siggaard Andersen, O.: A graphic representation of changes of the acid-base status. Scandinav. J. Clin. & Lab. Investigation *13,* 296, 1960.

— O. & Engel, K.: A new acid-base nomogram. An improved method for the calculation of the relevant blood acid-base data. Scandinav. J. Clin. & Lab. Investigation *12,* 177, 1960.

— O., Engel, K., Jørgensen, K. & Astrup, P.: A micro method for determination of pH, carbon dioxide tension, base excess and standard bicarbonate in capillary blood. Scandinav. J. Clin. & Lab. Investigation *12,* 172, 1960.

Singer, R. B., Shohl, J. & Bluemle, D. B.: A modification of the Shock & Hastings technique for the simultaneous determination of pH, CO_2 content and cell volume in 0.1 ml aliquots of cutaneous blood. Clin. Chem. *1,* 287, 1955.

Yoshimura, H.: Effects of anticoagulants on the pH of the blood. J. Biochem. *22,* 297, 1935.

Scandinav. J. Clin. & Lab. Investigation 205—211, 13, 1961.

FACTORS AFFECTING THE LIQUID-JUNCTION POTENTIAL IN ELECTROMETRIC BLOOD pH MEASUREMENT [1]

By O. SIGGAARD ANDERSEN

From the Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark.

(Received for publication September 13, 1960)

One of the problems concerning electrometric pH determinations is the often unknown junction potential arising at the liquid-junction between the salt bridge and the solution to be measured. This junction potential need not be the same with the standard buffer and the test solution, and the difference between the two is called the residual junction potential. It is evident that the junction potential influences the reproducibility and stability of electrometric measurements. Several factors are known to influence the junction potential *i. e.*: the physical structure of the junction; the salt solution in the bridge; the temperature of the junction, and colloids. If the residual junction potential is affected by these factors then the measured pH value in the test solution is altered. It is the purpose of this paper to show how these factors may influence the measured pH value of blood or plasma.

METHODS

A direct reading pH meter of the type PHM 22 (Radiometer) accurate to about ± 0.001 pH units was used.

The electrode set comprised a capillary glass electrode and a saturated calomel electrode as described by Siggaard Andersen, Engel, Jørgensen & Astrup, 1960. The glass capillary, length 10 cm, capacity 20 µl, is manufactured from Corning. 015 glass. The liquid-junction is formed between the test solution in a polyethylene tube (0.1—0.5 mm diameter) dipping into the salt bridge in a vessel kept at a constant temperature ("thermostated"). Connexion with the calomel cell is provided by a porous plug in the bottom of this vessel.

The standard buffer was a 1/15 M phosphate buffer prepared in large batches from 1.8156 g KH_2PO_4 (Sørensen) and 9.4934 g $Na_2HPO_4,2H_2O$ (Sørensen) in 1000 ml aqueous solution. The pH value for each batch was measured accurately at 38° C against a certified phosphate buffer with a pH value of 7.381 according to the NBS scale (Baumwoll Spinner & Petersen, 1961). The values of the batches measured in this way were always very close to 7.370.

[1] This work was supported by grants from "The Danish Foundation for the Advancement of Medical Science" and "The Danish State Research Foundation".

RESULTS

A) *Stability of the pH-readings.*

The stability of the electrode system was investigated by filling the glass electrode with the sample and following the pH-reading for

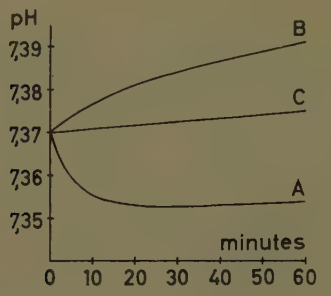


Fig. 1. The drift of the phosphate buffer pH reading at 38° C when the electrode has been stored for some days before the experiment at 38° C in 0.1 N HCl (A); in 0.01 M borate buffer (pH 9.09) (B), and 1/15 M phosphate buffer (pH 7.37) (C).

a half to one hour. A more or less pronounced drift was observed depending on the pH at which the glass electrode had been stored before the measurement. Fig. 1 shows how the buffer pH-reading changes in the course of one hour when the electrode was stored for some days at 38° C with 0.1 N MCl; a borate buffer (pH 9.09) or the phosphate buffer (pH 7.37). It is seen that the pH-reading transiently drifts to the acid side when the electrode has been stored in acid but otherwise drifts to the basic side.

The drift was further found to depend on the buffering capacity of the sample. When the electrode was stored with the 7.37 buffer the following drifts were found at 38° C: with distilled water from, 7.21 to 7.39 in 10 minutes, to 9.5 in 24 hours; with 20 mM sodium-acetate plus some HCl, from 7.41 to 7.47 in 10 minutes; with 20 mM sodium bicarbonate (with a pCO_2 approximately 40 mm Hg), from 7.420 to 7.441 in one hour with phosphate buffer from 7.370 to 7.375 in one hour. Different electrodes were used and the values quoted refer to the one which showed the least drift.

The following possibilities were considered as a cause of this drift:

1) Change in the normal potential of the electrode showing itself as an altered buffer adjustment before and after the experiment. When the electrode was stored with 0.1 N HCl the initial drift to the acid side of the buffer pH was explained by a change in buffer adjustment. In all other cases the buffer adjustment was constant within $\pm$ 0.005 pH.

2) Change in the junction potential was investigated by following the drift for 10 to 15 minutes after which a new liquid junction was made, either by cutting off about 1 cm of the plastic tube dipping into the salt bridge or by exchanging the plastic tip for a new one filled with the sample. Independent of the sample used (pure solutions or plasma) the reading always changed less than $\pm$0.003 in pH by this procedure. The junction potential thus seems very reproducible and stable within one hour.

3) Loss of CO_2 from the ends of the electrode could increase the basic drift in solutions containing CO_2. Replacement of the plastic tip with a glass tip, together with closure of the open end of the electrode by means of a rubber band, did not change the drift measurably. Nevertheless the end

electrode was always closed during in-
tigations of the electrode drift.

) Diffusion from the thermostated part of
electrode to the unthermostated ends
ld theoretically give rise to some drift.
h the whole electrode at the same tempe-
ıre (room temperature) some drift was
present; but much less than at 38° C.

) Solution of the electrode glass with
sequent discharge of base to the sample
y exclusion regarded as the main cause
he basic drift. In favor of this view is the
that the drift is very dependent on the
fering capacity of the sample.

Physical structure of the liquid-junction.

he importance of the diameter of the
ethylene tip was investigated by filling
electrode with sample and then alter-
ly measuring with a wide tip (0.7 mm
r diameter) and a narrow tip (approx-
tely 50 μ inner diameter). No significant
erence in pH could be measured either
ı buffer or whole blood. The readings
ed within ± 0.003 with both types of
id junction. It was found to be unim-
ant whether the plastic tip was placed
within the middle of the salt bridge or
against the side of the vessel.
matter of some importance was the sedi-
tation of the red cells out of the plastic
through the liquid junction. This some-
s occurred with the wide tip giving rise
ınstable readings. Most often, however,
precipitation of the proteins by the
rated KCl gave rise to a sharp, well
ıed junction.

C) *Composition of the salt bridge.*

Salt bridges of saturated KCl, 0.15 M KCl and 0.15 M NaCl were prepared by filling the small thermostated vessel in the calomel electrode with the respective salt solutions. Measurements were made at 38°C. The pH-value of the phosphate buffer was adjusted to 7.370 in each type of bridge and the pH-value of the plasma sample was measured against this. Three different normal plasma samples were used; each measurement repeated 4 times. With a 0.15 M KCl bridge the plasma pH was found to be 0.105 lower than with the saturated KCl bridge (range 0.102—0.108). With a 0.15 M NaCl bridge, the plasma pH was found to be 0.136 (0.132—0.140) lower than with the saturated KCl bridge.

D) *Temperature of liquid-junction.*

During the investigation of the significance of the temperature of the liquid-junction, the thermostated calomel electrode was replaced by a non-thermostated electrode (K 100, Radiometer). A small beaker with saturated KCl constituted the salt bridge. The beaker was either cooled or warmed and the temperature accurately measured simultaneously with the pH-measurement. The buffer pH was adjusted to 7.370 at each temperature and the plasma pH read against that. Fig. 2 shows the results. It is seen that the plasma pH decreases 0.0010 for each degree centigrade the temperature of the liquid junction increases. With a 0.15 M KCl bridge a decrease of 0.0015 pH units per degree centigrade was found.

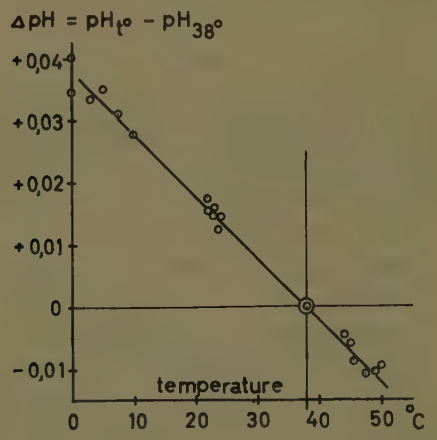


Fig. 2. Effect of temperature of the liquid-junction (saturated KCl) on the pH value of plasma measured against a phosphate buffer adjusted to 7.37 at any temperature. The ordinate shows the difference between the pH value measured at a given temperature and the value measured at a temperature of 38° C.

E) *"Suspension effect" of the red cells".*

pH measured at 38° C on whole blood was compared with the pH measured at 38° C on plasma and a concentrated red cell suspension prepared by anaerobic centrifugation of the blood at 38° C for about 5 min. Five experiments were performed with salt bridges of saturated KCl, 0.15 M KCl and 0.15 M NaCl. The mean value and the range of the results is shown in Table I. It is seen that a saturated KCl bridge gives more acid pH values in whole blood and concentrated cell suspensions than in plasma, 0.010 and 0.040 respectively. With bridges of 0.15 M KCl or NaCl this difference practically disappears. This indicates that

Table I. *Suspension effect of red cells.*

Salt bridge	Δ pH whole blood	Δ pH conc. cells
sat. KCl	— 0.010 ± 0.005	— 0.040 ± 0.011
0.15 M KCl	— 0.001 ± 0.007	— 0.005 ± 0.010
0.15 M NaCl	+ 0.002 ± 0.006	— 0.003 ± 0.009

pH values of plasma, whole blood and concentrated red cells were measured at 38° C after anaerobic separation at 38° C by centrifugation for 5 minutes. The temperature of the junction was 38° C. The hematocrit value of the concentrated cell suspension was about 90—95 per cent. $pH = pH_{blood} - pH_{plasma}$ was calculated. The table shows mean value and range of results of 5 determinations.

Table II. *Red cell effect on junction potential.*

Salt bridge	Δ pH whole blood	Δ pH conc. cells
sat. KBl	— 0.010 ± 0.003	— 0.036 ± 0.
0.15 M KCl	— 0.001 ± 0.003	— 0.005 ± 0.
0.15 M NaCl	0.000 ± 0.002	0.000 ± 0.

The effect on the pH value of plasma of the in polation of a bridge of whole blood or concentr cell suspension (HTC approximately 90—95 cent) measured at 38° C. The temperature of junction was also 38° C. The mean value and ra of results from 5 determinations is shown.

red cells do not affect the glass membr nor the junction potential, when unhe lyzed.

To illustrate this further the following periments were performed. The electr was filled with plasma and the pH was with the plastic tip filled with either plas whole blood or concentrated cell suspens Five experiments performed with each of salt bridge. Table II shows the m value and the range of the results. It is that practically the same values for "suspension effect" as reported in Tab

found when the red cells affect the junct- potential only.

nvestigation of the suspension effect with liquid junction at room temperature gave htly greater values: whole blood being 18 more acid than plasma.

DISCUSSION

Stability of the pH-reading.

The spontaneous drift to the basic side of pH readings found for solutions with pH ues about 7 to 8 was concluded to be sed by solution of the electrode glass, with charge of base to the sample. As there is sort of stirring, the discharged base may nge the pH in a thin layer close to the ss membrane.

t is well known that electrode glass diss- es in water, especially at pH-values above . This was shown by Hubbard, Hamilton Finn (1939), who further showed that the ss swells at pH-values below 7; probably to water imbibition of the silicate net- k.

The present investigations indicate that least drift is obtained if the electrode is red at a pH value close to the values the trode is expected to measure. However, he electrode is out of use for longer per- s it should probably be kept at acid pH —6), to diminish the solution of the elec- le glass.

Physical structure of the liquid-junction.

Several autho rs have drawn attention to significance of the structure of the liquid ction (Bates, 1954; Wright, 1959). Both authors emphasize the necessity of cylindrical symmetry to obtain the highest reproducibility and stability. Wright finds a three to four-fold increase in the drift if the polyethylene tip lies against the side of the vessel. The present investigations have not confirmed this finding. It possibly makes a diff- ence whether the polyethylene tube is filled with the sample or the salt bridge. The latter was the case in Wright's investigations.

C). *Composition of the saltbridge.*

The use of saturated KCl as bridge is nearly universal as this is believed to give minimal junction potentials. Wright (1959) and Mattock (1959) have recently recommended the use of an isotonic bridge solution for blood pH measurements from the consideration that saturated KCl precipitates proteins at the junction, which causes irreproducibility and instability. No data have been found in the literature for the comparison of pH values of plasma measured with different salt bridges. The present data show that the biological pH scale would change considerably if it was decided to use either physiological KCl or physiological NaCl as bridge solution; namely —0.105 and —0.136 respectively.

D) *Temperature of liquid-junction.*

It is well known that the junction potential changes with the temperature of the junction. If, however, the junction potential changes in the same way for the buffer and the plasma so that the residual junction potential is constant then the temperature of the junction

is insignificant because the pH value of the buffer is always adjusted before measurement of the sample. The present data show, however, that the residual junction potential changes considerably, so that a plasma pH value measured with the liquid junction at room temperature is approximately 0.015 more basic than the value measured with the junction at 38° C when a saturated KCl bridge is used. This shows the necessity of keeping the junction at a constant temperature to obtain comparable and reproducible results.

E) *"Suspension effect" of red cells.*

It was shown by Jenny, Nielsen, Coleman & Williams (1950) that the measured pH is often too low in colloidal solutions due to the effect of colloidal particles on the junction-potential. In 1956 Severinghaus, Stupfel & Bradley showed that the pH value measured on whole blood was 0.008 lower than the pH value measured on the corresponding plasma. The present data are in very good agreement with this finding as the difference between whole blood pH and plasma pH is found to be 0.010 when a salt bridge of saturated KCl of 38° C is used. It is shown that the effect is due to precipitation and haemolysis of the red cells at the junction causing an altered junction potential. Intact cells apparently do not affect either the junction potential or the glass membrane.

The consequence of the difference between whole blood pH and plasma pH when using a saturated KCl bridge seems to be the use of a physiological saline bridge (either KCl or NaCl) as proposed by Wright and Mattock. This involves, however, a considerable change in the biological pH sca and thereby creates a series of new problem which appears greater than the present.

Another possibility would be always separate the plasma anaerobically at 38° This would further eliminate problems d to glycolysis in the blood, but it would i volve certain technical difficulties.

The most practical solution at present possibly to retain the saturated KCl brid and then distiguish between whole blood p and plasma pH, estimating the difference be 0.010.

SUMMARY

Different factors affecting blood pH mea urement with the capillary glass electro were investigated.

The pH reading tended to drift to t basic side for solutions with pH values abo 7—8. This drift is considered to be due solution of the electrode glass with su sequent discharge of base to the test soluti

The junction potential did not drift mea urably within half an hour and was ind pendent of the diameter of the liquid jun ion.

The residual junction potential varied w the type of salt bridge and the temperature the junction.

Intact red cells were apparently with effect on the junction potential or the gl membrane, but precipitated and hemoly red cells at a saturated KCl-junction chang the junction potential so that whole blod is 0.010 lower than plasma pH, when junction has a temperature of 38° Ce grade.

REFERENCES

tes, R. G.: Electrometric pH Determinations. Theory and Practice. John Wiley & Sons, Inc., New York, 1954.

umwoll Spinner, M. & Petersen, G. Kokholm: Determing the pH of a phosphate buffer solution for blood measurements. Scandinav. J. Clin. & Lab. Investigation *13,* 1, 1961.

bbard, D., Hamilton, E. H. & Finn, A. N.: Effect of the solubility of glass on the behaviour of the glass electrode. J. Research Natl. Bur. Standards *22,* 339, 1939.

ny, H., Nielsen, T. R., Coleman, N. T. & Williams, D. E.: Concerning the measurement of pH, ion activities, and membrane potentials in colloidal systems. Science *112,* 164, 1950.

Mattock, G.: Electrochemical aspects of blood pH measurements. Woolmer, R. F.: A symposium on pH and blood gas measurement. Methods and interpretations. J. & A. Churchill Ltd., London 1959, 19—29.

Severinghaus, J. W., Stupfel, M. & Bradley, A. F.: Accuracy of blood pH and pCO_2 determinations. J. appl. Physiol. *9,* 189, 1956.

Siggaard Andersen, O., Engel, K., Jørgensen, K., & Astrup, P.: A micro method for determination of pH, carbon dioxide tension, base excess and standard bicarbonate in capillary blood. Scandinav. J. Clin. & Lab. Investigation *12,* 172, 1960.

Wright, M. P.: pH measurements with the glass electrode. Woolmer, R. F.: A symposium on pH and blood gas measurement. Methods and interpretations. J. & A. Churchill Ltd., London 1959, 5—15.

Scandinav. J. Clin. & Lab. Investigation 212—215, 13, 1961.

THE PARTICULATE NATURE OF INTRINSIC PROTHROMBINASE

By O. HUSOM

From the Institute for Thrombosis Research, University Hospital, Oslo, Norway.

(Received for publication September 15, 1960)

The prothrombin conversion principle in the extrinsic blood clotting system, extrinsic prothrombinase, is formed during incubation of tissue thromboplastin, proconvertin reagent, (containing factor VII and factor X) calcium chloride and accelerin. The active principle can be sedimented at high speed centrifugation and is thus bound to particles (Hjort, 1957).

A similar activity in the intrinsic blood clotting system, intrinsic prothrombinase, is formed on incubating serum, adsorbed plasma, platelet suspension and calcium chloride as shown by Biggs, Douglas & Macfarlane (1953). A cephalin suspension can be substituted for platelets (Bell & Alton 1954).

Bergsagel & Hougie (1956) obtained an active clotting principle in the sediment when they centrifuged an incubation mixture containing normal serum, factor VIII, calcium and platelets. The activity of the supernatant was not mentioned. Since all known clotting factors were present in this incubation mixture, the activity may be due, at least in part, to a fully developed intrinsic prothrombinase and not to an earlier intermediate. If this is so, they showed that the intrinsic prothrombinase is sedimentable, possibly bound to platelet granules.

Streuli (1959) states that the deposit afte high speed centrifugation of the thrombo plastin generation mixture, when dissolve has the same thromboplastic activity as tha of the incubation mixture. A chlorofor brain extract was used instead of platelet He does not mention the activity of th supernatant.

It is the purpose of this report to sho that the *main activity* of intrinsic prothrom binase can be sedimented whether platelets a cephalin suspension is used in the incuba ion mixture.

MATERIALS

Adsorbed bovine plasma. Barium sulphate fo lowed by aluminum hydroxide was used accordi to the method of Wagner, Richardson & Brink hous (1957). This plasma contains factor VIII b only very small amounts of fibrinogen. The co tent of factor V is low. The plasma was stor liquid frozen and was diluted immediately befo use.

Buffer. A modified veronal buffer (pH 7.35 a ionic strength 0.154) was used (Owren 1947).

Buffer-saline. A mixture of one-tenth volume buffer in saline was used.

Calcium chloride 30 mM.

Cephalin. A crude cephalin suspension was pre-
red from human brain as described by Hjort,
apaport & Owren (1955). A 1:200 dilution in
ffer of this stock suspension was used.

Dilution fluid II was prepared as described by
jort *et al.* (1955).

Fibrinogen. Bovine fibrinogen was prepared by
e method of Kekwick & MacKay (1954). It was
ored at —20° C.

Normal citrated platelet-poor plasma. Human
ood was collected in one-tenth volume of 3.13
n per cent sodium citrate dihydrate and centri-
ged for 30 minutes at 2,500 r.p.m. The plasma
as pipetted off and stored at —20° C.

Normal serum was prepared according to the
ethod described by Waaler (1959). It was stored
—20° C and was diluted immediately before use.

Platelet suspension was prepared according to
e method described by Biggs *et al.* (1953).

Proaccelerin reagent was prepared by the method
Owren (1947). This reagent is free of prothrom-
n. It contains small amounts of factor VIII.

METHODS

Intrinsic prothrombinase was prepared by incu-
tion of the following mixture: 0.6 ml normal
rum diluted 1:10 in dilution fluid II + 0.6 ml
lsorbed bovine plasma diluted 1:5 in dilution fluid
+ 0.2 ml proaccelerin reagent + 0.6 ml platelet
spension *or* cehpalin suspension + 0.6 ml calcium
loride. After incubation at 37° C for 8 minutes,
e mixture was centrifuged for 15 minutes at
,000 r.p.m. The supernatant was decanted off,
d the sediment (traces) was dissolved in 0.5 ml
ffer-saline.

The sediment and supernatant were tested in
plicate at 37° C in the following systems.

a) For intrinsic prothrombinase:

0.2 ml normal citrated platelet-poor plasma +
2 ml buffer-saline + 0.2 ml calcium chloride + 0.2
l test material. Calcium chloride was followed
ithin 5 seconds by the test material.

b) For thrombin:

0.2 ml fibrinogen + 0.2 ml buffer-saline + 0.2
l test material.

RESULTS AND COMMENTS

As shown in the table, the coagulant activity of the sediment versus plasma is very high and much higher than that of the supernatant. The activity of the sediment in the amount of suspension fluid used is about equal to the activity of the incubation mixture. The thrombin activity of sediment and supernatant is very low, and there is little difference between them. The high activity of the sediment must therefore be due to intrinsic prothrombinase. Thus the prothrombinase is sedimentable and must be bound to particles.

The extent of sedimentation was estimated by comparing the activity of the supernatant with the activity of the noncentrifuged incubation mixture. The incubation mixture was tested in different dilutions. When clotting time of dilutions was plotted on double logarithmic scale, a linear relationship was found. If the concentration of intrinsic prothrombinase in the incubation mixture was chosen as the one hundred per cent value, the concentration of prothrombinase in the supernatant was 2—5 per cent as read from the correlation graph. Thus 95—98 per cent of the prothrombinase in the incubation mixture was sedimented.

The activity of the sediment and the supernatant was of the same order of magnitude whether platelets or cephalin were used. Bergsagel & Hougie demonstrated that Product I (serum factor(s) + factor VIII + calcium) interacts with platelet granules and not with whole platelets or platelet fragments. These granules are probably identical with the platelet lipoid factor. Intrinsic prothrombinase may thus be sedimented with lipoid aggregates from the platelets.

Table I. *Intrinsic prothrombinase and thrombin in sediment and supernatant following high-speed centrifugation of the incubation mixture (see text).*

Source of lipoid factor	Clotting time in seconds				
	Substrate: Normal plasma			Substrate: Fibrinogen	
	Incubation mixture	Sediment	Supernatant	Sediment	Supernatant
Platelet suspensjoo	7.5	6.8	28.4	380	420
Cephalin suspensjon	8.0	7.8	34.2	460	540

It is conceivable that the function of the cephalin particles is similar to that of the platelet granules. Sedimentation rate of the cephalin particle parallel to that of intrinsic prothrombinase would indicate that this might be so. When a cephalin suspension was centrifuged at 25,000 r.p.m. for 15 minutes, cephalin was sedimented as a small white pellet, and the supernatant was clear. The degree of sedimentation was estimated in the same incubation mixture used in the production of intrinsic prothrombinase except that buffer replaced calcium chloride. After centrifugation at the same speed the different layers and the sediment were tested in the following system, which is sensitive to cephalin: 0.2 ml normal platelet-poor plasma from a fasting individual + 0.1 ml Russel's viper venom diluted 1 : 20,000 + 0.2 ml test substance + 0.2 ml calcium chloride 30 mM. The non-centrifuged mixture gave a clotting time of 10.6 seconds. After centrifugation, identical clotting times at 18.6 seconds were recorded from the top, middle and bottom layers. The sediment suspended in 0.5 ml of buffer gave 8.7 seconds. The non-centrifuged mixture was tested in different dilu tions. In dilution 1:6 the clotting time wa 17.6 seconds. From this it was calculate that about 95 per cent of the cephalin par ticles were sedimented. Thus, when cephali is used in the production of intrinsic pro thrombinase, they are both sedimented to comparable degree. Whether cephalin chemically involved in the complex or act by the physical property of its particle siz is not known.

SUMMARY

Intrinsic protrombinase is prepared wit serum, adsorbed plasma containing onl traces of fibrinogen, proaccelerin reagen calcium and platelet suspension *or* cephali Intrinsic prothrombinase is sedimented to very high and about equal degree in bot instances and is thus bound to particles. Th sedimentation seems to run parallel to tha of cephalin, suggesting that intrinsic pro thrombinase may be bound to the cephali particles, which seem to be equivalent to th platelet granules.

REFERENCES

l, W. N. & Alton, H. G.: A brain extract as a substitute for platelet suspensions in the thromboplastin generation test. Nature *174,* 880, 1954.

gsagel, D. E. & Hougie, C.: Intermediate stages in the formation of blood thromboplastin. Brit. J. Haemat. *2,* 113, 1956.

gs, R., Douglas, . S. & Masfarlane, R. G.: The formation of thromboplastin in human blood. J. Physiol. *119,* 89, 1953.

Douglas, A. S.: The thromboplastin generation test. J. Clin. Path. *6,* 23, 1953.

ort, J., Rapaport, S. I. & Owren, P. A.: A simple, specific onestage prothrombin assay using Russell's viper venom in cephalin suspension. J. Lab. & Clin. Med. *46,* 89, 1955.

ort, P. F.: Intermediate reactions in the coagulation of blood with tissue thromboplastin. Convertin, accelerin, prothrombinase. Thesis. Scandinav. J. Clin. & Lab. Investigation *9,* Suppl. *27.* 183, 1957.

Kekwick, R. A. & MacKay, M. E.: The separation of protein fractions from human plasma with ether. Spec. Rep. Ser. Med. Res. Coun. (Lond.) *286,* 75, 1954.

Owren, P. A.: The coagulation of blood. Investigations on a new clotting factor. Thesis. Acta med. Scandinav. *128,* Suppl. 194, *327,* 1947.

Streuli, F.: On the purification and conversion of human prothrombin. Thromb. Diath. haem. *3,* 194, 1959.

Waaler, B. A.: Contact activation in the intrinsic blood clotting system. Studies on a plasma product formed on contact with glass and similar surfaces. Thesis. Scandinav. J. Clin. & Lab. Investigation *11,* Suppl. *37,* 133, 1959.

Wagner, R. H., Richardson, B. A. & Brinkhous, K. M.: A study of the separation of fibrinogen and antihemophilic factor (AHF), in canine, porcine, and human plasma. Thromb. Diath. haem. *1,* 3, 1957.

Scandinav. J. Clin. & Lab. Investigation 216—224, 13, 1961.

INTRINSIC PROTHROMBINASE: INVESTIGATIONS ON THE POSSIBILITY OF ITS FORMATION BY SUCCESSIVE COATING OF INTERMEDIATES ON ACTIVATED SURFACES

By O. HUSOM

From the Institute for Thrombosis Research, University Hospital, Oslo, Norway.

(Received for publication September 15, 1960)

In the intrinsic blood clotting system at least eight clotting factors participate in the production of the prothrombin conversion principle (intrinsic prothrombinase, blood thromboplastin). They are the Hageman factor, the antihemophilic C factor (P.T.A.), factor V (proaccelerin), factor VIII (antihemophilic A factor), factor IX (antihemophilic B factor), factor X (the Stuart factor), the platelet lipoid factor, and calcium. It is unlikely that all these factors react simultaneously, more probably the reactions occur stepwise.

The initial reaction on contact with foreign surface has recently been investigated by Margolis (1957), Ratnoff & Rosenblum (1957), Soulier, Wartelle & Ménaché (1958), Biggs, Sharp, Margolis, Hardisty & Stewart (1958), and Waaler (1959). There is general agreement that the Hageman factor and the antihemophilic C factor are involved in this reaction, although the views as to the mode of reaction differ.

Waaler observed that all normal and abnormal plasmas, except the Hageman d ficient and the antihemophilic C factor d ficient plasmas, clotted more rapidly aft shaking with celite, glass beads or quar powder. He showed that an active princip "the activation product", was formed duri this procedure. The activation product w firmly fixed to the powder used for activ tion, and its activity was not significant reduced by repeated washings. It was stab for months on storage at —20° C. He co cluded that the Hageman factor and the an hemophilic C factor are probably both some way incorporated in this product. T product does not contain calcium.

Activation product is the starting point f the subsequent intermediate reactions. Io ized calcium is needed at least for some these reactions. It is conceivable that t reactions take place on the surface of t particles which carry the activation produ If so, the intermediates may either be r leased from the particles, or continue to sti to the surface as coatings. The last pos

lity suggests a means for the isolation and udy of intermediates, since the coated part-es may easily be separated at any stage in e chain of reactions. The present invest-ation is a study along this line of approach. similar approach has been fruitful in the udy of intermediates in the extrinsic clot-ng system (Hjort 1957). However, the re-lts show that the particles coated by activ-ion product do *not* behave as nucleus for atings by successive intermediates in the trinsic clotting system.

MATERIALS AND METHODS

Activation product was prepared by the method scribed by Waaler (1959). Normal oxalated man plasma adsorbed with barium sulphate, 100 per ml of plasma was used for activation. e adsorbed plasma was shaken with 1/4 the ume of celite. The powder was removed by ntrifugation, washed three times with buffer-ine and resuspended in the original plasma ume of buffer-saline. The reagent was stored uid frozen at —20° C. It contains small amounts factor V and VIII. Unless otherwise stated, s reagent was used as the source of activation oduct.

Buffer. A modified veronal buffer (pH 7.35 and ic strength 0.154) was used (Owren 1947).

Buffer-saline. A mixture of one part of buffer l nine parts of saline was used.

Calcium chloride 30 mM.

Celite powder. Speedex, which is amorphous tomaceous SiO_2 from Dicalite Divisions, Great kes Carbon Corp., New York was used.

Cephalin. A crude cephalin suspension was pre-ed from human brain as described by Hjort, paport & Owren (1955). A 1:200 dilution in fer of this stock suspension was used.

Dilution fluid II was prepared as described by ort *et al.* (1955).

Fibrinogen. Bovine fibrinogen was prepared by method of Kekwick & MacKay (1954). It s stored at —20° C.

Plasma.

a) Normal citrated human plasma. Blood was collected in one tenth volume of 3.13 gm per cent sodium citrate dihydrate and centrifuged for 30 minutes at 2,500 r.p.m. The plasma was stored at —20° C.

b) Exhausted human plasma was prepared according to the method of Waaler (1959). Plasma was shaken with celite powder. The powder was removed by centrifugation and the activated plasma stored at 37° C for 5—6 hours. This procedure inactivates the activation product, and the plasma is thus "exhausted" as regards this product. The other clotting factors are present in normal amounts except for a moderate reduction of factors V and VIII.

c) Adsorbed oxalated bovine plasma. Whole blood was collected in one tenth volume of 2.5 per cent potassium oxalate monohydrate and centrifuged for 30 minutes at 2,500 r.p.m.. The plasma was adsorbed with 100 mg of barium sulphate per ml of plasma for 5 minutes at room temperature and centrifuged for 30 minutes at 2,500 r.p.m. The plasma was dialyzed overnight against saline and stored at —20° C. In some experiments exhausted plasma of this batch was used. The adsorbed dialyzed plasma was then adjusted to pH 7.35 and activated with 1/4 the volume of celite. After centrifugation the supernatant plasma was kept at 37° C for 5 hours. It was then stored at —20° C.

d) Barium sulphate + aluminum hydroxide adsorbed bovine plasma was prepared according to a method described by Wagner *et al.* (1957). One ml of barium sulphate adsorbed plasma was adsorbed with 0.3 ml of aluminum hydroxide gel. The object is to remove fibrinogen without materially reducing the content of factor VIII. Factor V is considerably reduced in this reagent. In some experiments, this plasma was also exhausted before adsorption with aluminum hydroxide.

Proaccelerin reagent was prepared according to the method of Owren (1947). It contains small amounts of factor VIII.

Serum was prepared by the method described by Waaler (1959). In some experiments exhausted serum was used. It was prepared in the same way as that described for adsorbed bovine plasma.

Thromboplastin serum was prepared according to the method described by Hjort (1957). Normal human blood is clotted in the presence of brain thromboplastin, three volume per cent. Thromboplastin is removed by centrifugation at 40,000 r.p.m. for 60 minutes. The content of prothrombin in this serum is below one per cent. The other serum clotting factors are unchanged, compared to serum prepared without the addition of brain thromboplastin. Assay of factor X was performed by Dr. C. L. Johnston, Jr. in our laboratory, using factor X deficient plasma from R. Stuart as substrate. This plasma was kindly sent us in lyophilized samples by Dr. John B. Graham, University of North Carolina School of Medicine, Chapel Hill, N. C., U. S. A.

Incubation and clotting experiments are described in the appropriate tables.

RESULTS

Incubation experiments with activation product and groups of clotting factors.

1) *Serum and calcium chloride.*

Table I shows that incubation with calcium chloride alone does not increase the activity of the activation product.

A moderately increased activity develops when activation product is incubated with serum in the absence of calcium. A smaller increase is noted when exhausted serum is used. The higher activity attained with serum as compared with exhausted serum must be due to the different amounts of activation product in these sera.

The increased activity observed when calcium is present during incubation may be due to the formation of intermediates, possibly thrombin. The minute amounts of factors V and VIII in the activated celite powder may be responsible for the formation of intrinsic prothrombinase, which converts th small amounts of prothrombin in serum t thrombin. If non-adsorbed plasma was use for the activation with celite, the powder wa contaminated with minute amounts of pro thrombin. On incubation with serum an calcium a much more pronounced increase i activity was observed than noted in Table This is shown in Table II. This increase i activity was shown to be due partly, mayb entirely, to thrombin. Even when activate

Table I. *Incubation of stored serum and activation product.*

Activation product incubated with:	Clotting time in secs.	
	Sediment	Supernatant
Dilution fluid II	75.8	145.8
Dilution fluid II + calcium chloride....	77.5	147.4
Stored serum + buffer-saline	54.6	73.4
Stored serum + calcium chloride....	43.5	56.5
Stored exhausted serum + buffer-saline	63.1	95.6
Stored exhausted serum + calcium chloride .	51.4	84.6

Incubation mixture: 1 ml activated celite suspen sion +1 ml of serum diluted 1:5 in dilution fluid II +1 ml of calcium chloride 30 mM *or* 1 ml o buffer-saline. The mixture was incubated at 37° C for 12 minutes. The powder was removed by centri fugation at 3,000 r.p.m. for 10 minutes. The super natant was pipetted off. The powder was suspende in 1 ml buffer-saline, and both were immediatel tested in the following system: 0.2 ml exhauste plasma +0.2 ml cephalin suspension 1:200 +0.2 m test material +0.2 ml calcium chloride 30 mM. Th first two reagents were preincubated for 6 minute at 37° C. The prewarmed solution of calcium chlo ride was added followed within 5 seconds by th prewarmed test material and the stopwatch starte The formation of a solid clot was registered.

te prepared from the plasma of a patient h severe hemophilia A was used in the eriment, thrombin was still formed. ble II shows that the hemophilia B plas- is far from corrected to the same level as mal plasma. This speaks against any in- mediate reaction taking place between ivation product, factor IX and calcium.

To evaluate the influence of thrombin in experiments presented in Table I, throm- blastin serum was used. thus reducing pro- ombin to the lowest level possible. Table

ble II. *Incubation of stored serum and activation product prepared from non-adsorbed plasma.*

ctivation product ncubated with:	Clotting time in secs.	
	Normal plasma	Hemophilia B plasma
ution fluid II + ium chloride...	50	151.8
red serum + ium chloride...	29.9	81.3

e sediment was tested against normal plasma plasma from a patient with hemophilia B. The cedure was otherwise as described in Table I.

ble III. *Incubation of thromboplastin serum with activation product.*

Activation product incubated with:	Clotting time in secs.	
	Sediment	Supernatant
ution fluid II + alcium chloride....	76.2	140.8
red exhausted romboplastin serum + buffer-saline	55.5	63.2
red exhausted romboplastin serum - calcium chloride .	54.2	63.2

The procedure was as described in Table I.

Table IV. *Incubation of thromboplastin serum and cephalin with activation product.*

Activation product incubated with calcium chloride and:	Clotting time of sediment in secs.
Dilution fluid II + cephalin .	89.1
Thromboplastin serum + buffer-saline	52.8
Thromboplastin serum + cephalin.....	51.5

Incubation mixture: 1 ml activated celite suspension +1 ml thromboplastin serum diluted 1:5 in dilution fluid II (*or* 1 ml of dilution fluid II) +1 ml cephalin suspension 1:200 +1 ml calcium chloride 30 mM. The procedure was further as described in Table I. The test system was the same.

III shows that thromboplastin serum and calcium do not increase the activity beyond that of the non-calcified mixture. It may thus be concluded that no intermediate reaction takes place when activation product is incubated with calcium and the serum clotting factors.

2) *Serum, calcium chloride and cephalin.*

Table IV shows that the addition of cephalin to an incubation mixture of activation product, serum and calcium has no influence.

3) *Adsorbed bovine plasma and calcium chloride.*

Table V shows that no increased activity is obtained on recalcification of a mixture of activation product and adsorbed bovine plasma. As the activated celite is contaminated with small amounts of factor VIII, a possible intermediate reaction involving this factor might be concealed in this experiment. Therefore a similar experiment was carried

out with activation product prepared from adsorbed hemophilia A plasma. Table VI shows that no increased activity develops on recalcification. Thus, no intermediate r action takes place between activation pr duct factor V, factor VIII and calcium.

Table V. *Incubation of activation product with adsorbed bovine plasma.*

Activation product incubated with:	Clotting time in secs.	
	Sediment	Supernatant
Dilution fluid II + calcium chloride....	85.2	149.7
Adsorbed plasma + buffer-saline	58.2	132.9
Adsorbed plasma + calcium chloride....	57.7	139.1
Adsorbed exhausted plasma + buffer-saline	67.2	135.4
Adsorbed exhausted plasma + calcium chloride....	64.4	135.6

Incubation procedure was as described in Table I. Adsorbed bovine plasma was diluted 1:5 in dilution fluid II. The test system was as described in Table I.

Table VI. *Incubation of adsorbed bovine plasma, calcium chloride, and activation product, prepared from hemophilia A plasma.*

Activitation product incubated with:	Clotting time in secs.	
	Normal plasma	Hemophilia A plasma
Dilution fluid II + calcium chloride....	46.4	243.0
Adsorbed bovine plasma + buffer saline.....	45.2	112.0
Asorbed bovine plasma + calcium chloride	44.0	112.6

Adsorbed bovine plasma was diluted 1:5 in dilution fluid II. The procedure was as described in Table I. The test system was the same except that normal and hemophilia A plasmas taken with silicone technique were used as substrate.

4) *Adsorbed bovine plasma, calciu chloride and cephalin.*

No reaction could be demonstrated wh these reagents were incubated, as appea from Table VII.

5) *Serum, adsorbed bovine plasma, ceph lin and calcium chloride.*

Table VIII shows the effect of increasi amounts of celite on the formation and a sorption of intrinsic prothrombinase.

Table VII. *Incubation of activation pr duct, adsorbed bovine plasma and cephali*

Activation product incubated with calcium chloride and:	Clotting time in cecs.
Dilution fluid II + buffer-saline	89.3
Absorbed bovine plasma + buffer-saline	63.6
Absorbed bovine plasma + cephalin.....	60.7

The procedure was as described in Table IV. A sorbed bovine plasma was diluted 1:5 in dilut fluid II. The test system was the same.

amount of celite above 1/20 the total volu of the incubation mixture inhibited the fo mation of intrinsic prothombinase. With d creasing amounts of celite the formation w stable. The activity was then highest in t supernatant because celite was not availab in sufficient amounts for adsorption.

The suspension of sediment with maximu activity clots fibrinogen in 150—180 conds. If thromboplastin serum is used, t

ble VIII. *The influence of celite on the ormation of intrinsic prothrombinase.*

Amount of celite (Volume celite / Volume buffer)	Clotting time in secs.	
	Sediment	Supernatant
1/1	16.0	54.4
1/2	9.0	22.4
1/5	7.8	11.4
1/8	9.6	9.8
1/16	12.0	9.6
1/32	14.2	8.8
1/64	17.6	8.8

insic prothrombinase was prepared by incubat- 1 ml barium sulphate-aluminum hydroxide ad- ed bovine plasma diluted 1:5 in dilution fluid II 2 ml proaccelerin reagent +1 ml cephalin sus- ion 1:200 +1 ml suspension of celite listed in mn 1. The mixture was prewarmed to 37° C. and l calcium chloride 30 mM was added. The incu- on was kept on for 10 minutes, and the mixture then centrifuged at 3,000 r.p.m. for 10 minutes. supernatant was pipetted off, the sediment sus- led in 1 ml buffer-saline, and both were tested he following system: 0.2 ml normal citrated ma +0.2 ml buffer-saline +0.2 ml calcium ride 30 mM. +0.2 ml test material. The first reagents were preincubated for 3 minutes at C. Calcium chloride was added followed within conds by the test material and the stopwatch started.

vity of the sediment is nearly unchanged sus normal plasma, while it takes nearly an hour to clot fibrinogen (Table IX). us, the high activity must be due to in- sic prothrombinase and not to thrombin. e binding to the celite powder is loose. e activity is substantially reduced by one hing.

s intrinsic prothrombinase built up on the vated surface of the celite particle, or is ormed in the solution and then adsorbed? test this, activated celite was compared to non-activated celite in the incubation mixture. Table X shows that the activated celite carries a lower activity leaving more in the supernatant as compared with non-activated celite. This probably means that less intrinsic prothrombinase can be adsorbed because the adsorptive sites on the celite particle are blocked by the activation product. The blocking might be due to other substances ad-

Table IX. *The adsorption of intrinsic prothrombinase and thrombin by celite using thromboplastin serum in the test.*

Sediment suspended in buffer-saline	Clotting time	
	Normal plasma	Fibrinogen
1 ml.	8.1 secs.	27 minutes
0.5 ml.	6.0 secs.	28.5 minutes

The incubation mixture was the same as in Table VIII, except that thromboplastin serum was used. It was diluted 1:10 in dilution fluid II. The suspension of celite added was 1:4, 1 ml. Two tests were run simultaneously. In one the sediment was suspended in 1 ml of buffer-saline, in the other 0.5 ml. The test system for thrombin was: 0.2 ml fibrinogen +0.2 ml buffer-saline +0.2 ml test material.

Table X. *The behavior of activated and non-activated celite during formation of intrinsic prothrombinase, using activated reagents.*

Type of celite	Clotting time in secs.	
	Sediment	Suerpnatant
Activated	11.9	8.3
Non-activated.....	7.0	19.7

The procedure was as described in Table VIII. Celite was used in suspension 1:4, 1 ml.

Table XI. *The influence of the amount of activation product in the celite suspension on the adsorption of intrinsic prothrombinase.*

Type of celite powder	Clotting time in secs.	
	Sediment	Supernatant
Activated with normal adsorbed human plasma	14.4	10.3
Activated as above and stored for 24 hrs. at 37° C	9.3	11.1
«Activated» with exhausted adsorbed human plasma	8.2	11.2
Non-activated	7.4	17.4

The incubation mixture and test system were as described in Table VIII. The celite suspensions in column 1 were 1:4, 1 ml.

sorbed in small amounts from the plasma. The results of the experiments shown in Table XI, however, support the view that the activation product is the substance responcible.

In the experiments presented in Tables VIII—XI, activation product is present both in the serum and plasma reagent and partly on the celite powder, in excess. Intrin prothrombinase might be formed both in t solution followed by adsorption and on t activated powder by stepwise building u To test what mode of action was taki place or essentially preferred, exhaust plasma and serum reagents were used in t test. Activated celite was the essential sour of activation product. Table XII shows th the intrinsic prothrombinase activity on t powder is higher when exhausted reagen are used as compared with activated r agents. In the supernatant the reverse found. This can be explained in the way th activation product is removed from the s face of the particles into the solution whe it reacts with other components to form i trinsic prothrombinase, which is then a sorbed to the powder, where more a sorptive sites are now available.

DISCUSSION

The activation product is the first inte mediate in the intrinsic blood clotting sy tem. It can be firmly bound to the cel particle, and this activated particle w

Table XII. *The distribution of intrinsic prothrombinase and thrombin between sediment and supernatant using activated celite and different amounts of activation product in the serum reagent.*

Type of serum	Clotting time in secs.			
	Normal plasma		Fibrinogen	
	Sediment	Supernatant	Sediment	Supernatant
Exhausted	8.0	13.0	153	16 minutes
Activated	11.3	8.3	182	16 minutes

Barium sulphate-aluminum hydroxide adsorbed bovine plasma and proaccelerin reagent were exhausted. The procedure was as described in Table VIII. The test systems were as described in Table VIII and IX.

refore used as a presumptive anchor nt for succeeding intermediates in the sent investigations. Activation product is med in the absence of calcium, but cal- m is needed during the following re- ions.

The clotting factors in serum which are ded for the reactions in the intrinsic sys- n are factors IX and X and possibly the phase accelerator of Fisch (PPA). No ction seemed to occur between these tors and the activation product in the pre- ce of calcium. Addition of cephalin to the xture had no effect.

Neither could a reaction be demonstrated en adsorbed plasma, containing factors V d VIII, was incubated with the activation duct, calcium and cephalin.

This is in accordance with the findings of rgsagel & Hougie (1956). They used telet granules as carrier for possible in- mediates, which could then be centrifuged t at high speed centrifugation. Serum, tor V or antihemophilic globulin sepa- ely incubated with calcium and platelets re essentially inactive. In the light of pre- t knowledge we may sag that the activation duct was present in their reagents. Both ite ant platelet-granules are of particulate ture. But the lipoids of the platelet granu- are probably chemically involved at some ge in the chain of reactions related to the rinsic blood clotting system, while the ite particle itself is intert in this respect. e activated celite particle is located at one d of the chain, the lipoid particle more to- rds the centre or the other end.

For intermediate reactions to occur, both um farctor(s) and factor(s) from ad- bed plasma must be present. When these factors are available together with cephalin and calcium, intrinsic prothrombinase is formed. Intrinsic prothrombinase is probably itself of particulate nature, but it needs a higher centrifugal force than the celite particle to be sedimented. The experiments show that intrinsic prothrombinase is loosely adsorbed to celite. If the celite particle is activated, it holds less intrinsic prothrombinase than in the inactive state, provided the other reagents contain the activation product. This probably means that the intermediate reactions following the formation of the activation product preferrably take place *outside* the celite particle and that formed intrinsic prothrombinase occupies adsorptive sites on the particle, where activation product is not present. The experiments with activated celite and exhausted reagents seem to show that the activation product is released from the particle into the surrounding solution where it reacts to form intrinsic prothrombinase which is then adsorbed. Thus the activated celite particle cannot be used as a nucleus for the building up of intrinsic prothrombinase or intermediates firmly fixed to the particle as the activation product itself.

These investigations have not thrown light on the sequence of factors or the mode of their interactions in the intrinsic blood clotting system. Important advances in this field can probably not be achieved before the clotting factors are isolated in a much more purified form than is possible today. Complete elimination of prothrombin is highly desirable. Contamination with even small amounts of this factor has been a most disturbing complication in the present experiments.

SUMMARY

The possibility of isolating intermediates in the intrinsic blood clotting system by incubating activated celite powder with groups of clotting factors is investigated.

No reaction was detected between activation product, serum, cephalin, and calcium. Neither was adsorbed plasma when substituting serum effective. Reactions started when serum and adsorbed plasma were both present. Intrinsic prothrombinase was then formed.

Intrinsic prothrombinase is loosely adsorbed to celite. The amount adsorbed to non-activated celite is higher than that adsorbed to an equal amount of activated celite.

Evidence is presented that activation product is removed from the particle surface before it takes part in the subsequent reactions with other clotting factors. Therefore it is not likely that intermediates are bound to the activated surface, and thus they cannot be isolated in this way.

REFERENCES

Bergsagel, D. E. & Hougie, C.: Intermediate stages in the formation of blood thromboplastin. Brit. J. Haemat. *2,* 113, 1956.

Biggs, R., Sharp, A. A., Margolis, J., Hardisty, R. M., Stewart, J. & Davidson, W. M.: Defects in the early stages of blood coagulation: A report of four cases. Brit. J. Haemat. *4,* 177, 1958.

Hjort, P., Rapaport, S. I. & Owren, P. A.: simple, specific one-stage prothrombin ass using Russell's viper venom in cephalin su pension. J. Lab. & Clin. Med. *46,* 89, 195

Hjort, P. E.: Intermediate reactions in the co gulation of blood with tissue thromboplasti Thesis. Scandinav. J. Clin. & Lab. Investig *9,* Suppl. *27,* 183, 1957.

Kekwick, R. A. & MacKay, M. E.: The separati of protein fractions from human plasma wi ether. Spec. Rep. Ser. Med. Res. Coun. (Lond No *286,* 75, 1954.

Margolis, J.: Initiation of blood coagulation glass and related surfaces. J. Physiol. *13* 95, 1957.

Owren, P. A.: The coagulation of blood. Invest gations on a new clotting factor. Thesis. Ac med. Scandinav. *128,* Suppl 194, 327, 1947.

Ratnoff, O. D. & Rosenblum, J. M.: The role Hageman factor in the initiation of clotti by glass. J. Lab. & Clin. Med. *50,* 941, 195

Soulier, J.—P., Wartelle, O. & Ménaché, D Caractères différentiels des facteurs Hage man et P. T. A. Rôle du contact dans phase initiale de la coagulation. Rév. fra Ét. clin. biol. *3,* 263, 1958.

Waaler, B. A.: Contact activation in the intrins blood clotting system. Thesis. Scandinav. Clin. & Lab. Investigation *11,* Suppl. *37,* 133, 1959.

Wagner, R. H., Richardson, B. A. & Brinkhou K. M.: A study of the separation of fibrinog and antihemophilic factor (AHF) in canin porcine, and human plasma. Thromb. Diat haem. *1,* 3, 1957.

ıdinav. J. Clin. & Lab. Investigation 225—230, 13, 1961.

A MICRO METHOD FOR DETERMINATION OF BARBITURATES IN SERUM AS MERCURIC COMPLEXES

By B. ZAAR and A. GRÖNWALL

From the Department of Clinical Chemistry, University Hospital and Research Laboratories, Pharmacia Ltd., Uppsala, Sweden.

(Received for publication September 9, 1960)

A number of different methods are avail-e for the determination of barbiturates in logical material. The procedures currently d are based on the ultraviolet absorption these compounds (Broughton, 1956; uld & Hine, 1949; Goldbaum, 1952; ıs, 1950; Richterich, 1958). Other meth- suggested but applied only to a limited ent are gravimetric analysis (Fleury, 5), colorimetric procedures using com-ınds of barbiturates with certain metal s (Koppanyi, Dille, Murphy & Krop, 4; Zwicker, 1931), indirect determination the metal component in barbiturate-metal ıplexes (Björling, Berggren, Willman-ınsson, Grönwall & Zaar, 1958; Pfeil & dbach, 1955), and infrared spectrophoto-ric analysis (Levi & Hubley, 1956). ese last mentioned procedures have not n used more extensively because of their eriority in specificity and sensitivity when y are compared with ultraviolet adsorp-ı methods. Although the ultraviolet meth- in most respects satisfy high demands, y have certain limitations that present mselves, especially at determinations of concentrations.

Investigations have been carried out to develop a micro method that enables detection of very small amounts of barbiturates in biological material. For this purpose, the ability of these compounds to form complexes with certain metals has been investigated (Björling *et al.*, 1958); and the method described in this paper is based on the results thereof. In earlier methods, the complexes with copper or cobalt were used, but no real micro method was published until Pfeil & Goldbach's paper in 1955. Their method, however, is worked out for analysis of water solutions; and the metal-compound formed has qualities other than those described in the present paper.

Principle

Barbiturates are extracted with ether from serum absorbed in filter paper. The isolated barbiturate will react at slightly alkaline pH with a mercury(II) salt, and the complex formed is isolated from excessive mercury (II) by extraction with chloroform. The amount of mercury in the complex is determined with dithizone.

METHOD

Material

Test tubes 150 x 20 mm with ground stoppers.
Pipettes 1, 4, 5, and 10 ml.
Funnels.
Strips of filter paper 100 x 25 mm, Whatman No. 1, washed with ether.
Filter paper Munktell No. 00, diameter 3.5 cm.

Reagents

A. R. grade reagents are used.
Chloroform.
Ether.

Mercuric-bicarbonate solution: 0.50 g $Hg(NO_3)_2$ is dissolved in 50 ml dist. water and a few drops of conc. HNO_3 are added. 1 ml of this solution is diluted with 50 ml 0.1 M $NaHCO_3$. The solution is filtered to remove any precipitate formed.

Dithizone solution: 32 mg dithizone is dissolved in 1000 ml chloroform.

Procedure

The analysis is carried out on 1 ml serum or plasma. As reagent blank, 1 ml distilled water is taken and it passes all steps of analysis together with the sample. The serum is applied on four rolled-up filter paper strips placed in a test tube with ground stopper. 10 ml ether is added, and the extraction is carried out by turning the tube up and down for 30 seconds. Violent shaking should be avoided. The ether fraction is poured into another test tube and the extraction is repeated with a new portion of 10 ml ether. The ether fraction is evaporated to dryness in a water bath of approximately 50° C. It is better to evaporate after each extraction in order to avoid bumping of the solution and risking the loss of material. The residue in the tube is dissolved in 5 ml chloroform; 0.5 ml mercuric-bicarbonate solution is added; and the tube is stoppered and shaken for 1/2—1 minute. After the separation of the phases, the chloroform layer is removed by a pipette and filtered through a Munktell No. 00 filter paper. For the colorimetric determination, 4 ml of the filtrate are taken and 1 ml dithizone solution is added. In the presence of mercury-barbiturate, the color is changed from emerald-green to orange, depending on the amount of mercury(II) present. The absorbance is read in 1 cm cell at 605 mμ against chloroform. The extinction difference between the reagent blank and the sample is a function of the barbiturate concentration, the numeric value of which is found from a standard curve made on known solutions. Alternatively, the extinction difference can be obtained directly by zero-balancing the instrument with the sample and reading the absorbance of the blank. If the concentration of mercury in the solution is so high that all dithizone is consumed, this will be evident from the pure orange color of the sample without any greenish-gray hue, 1 ml dithizone solution and 4 ml chloroform are added. In such a case the obtained concentration of barbiturate is multiplied by two.

DISCUSSION

Extraction of barbiturates from serum

In most methods chloroform is used for extraction of barbiturates from serum, and a number of investigations have been published concerning the recovery of barbiturates and the optimum conditions when using this solvent (Lous, 1950; Dybing, 1955; Hendorff, 1955). In the present method, ether has been chosen because of its lower boiling point and because of the more favorable distribution coefficient of barbiturates in ether as compared with chloroform.

The distribution ratio D for some barbiturates between ether and aqueous buffer solution of pH 7.4 has been determined. For isoamyl-ethyl-barbituric acid, D = 33.8; for phenyl-ethyl-barbituric acid, D = 8.9; and for diethyl-barbituric acid, D = 3.9. Diethyl-barbituric acid is extracted to 97 per cent when the volume relation between ether- and water-phases is 10 to 1.

n normal serum and plasma, there can ur ether or chloroform extractable or- ic compounds which react with mercuric s thereby giving a certain blank value. It ms possible to reduce this interference absorption of serum in filter paper before raction and this even facilitates the ex- ction procedure. Two extractions with 10 ether each are sufficient for quantitative overy of barbiturates.

rmation and extraction of mercury- biturate complexes

The mercury(II)-barbiturate complexes e not been thoroughly investigated, but y can exist in more than one form de- ding on the pH at which they are formed. the reaction between mercury(II) and biturates takes place in acid solution, a cipitate is formed which is insoluble in anic solvents. This phenomenon has earl- been utilized for the determination of biturates in Pfeil & Goldbach's method. the other hand, if the reaction proceeds slightly alkaline solution, a complex is ned which can be extracted with chloro- n and some other organic solvents. This plex formation and its extraction can ceed within quite a broad pH range, ap- ximately from pH 7 to 11 (Björling, ggren & Willman-Johnsson, 1959). For optimum recovery at extraction, however, concentration of water and chloroform st be kept within certain ranges. Fig. 1 ws the influence of the mercuric ion con- tration on extraction of diallyl-barbituric l. A large excess of mercury(II) has an ibitory effect on the extraction of small ounts of barbiturates. A certain difference ehavior between various barbiturates can

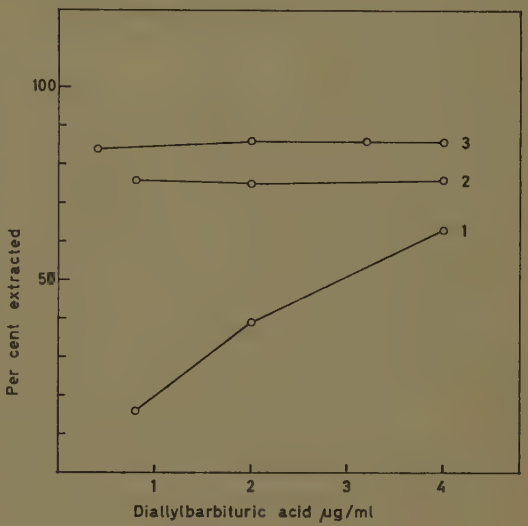


Fig. 1. The influence of mercury ion concentration in the water phase on the extraction of the diallyl-barbituric acid-mercury complex with 5 ml chloroform.

1. 400 µg Hg in 0.5 ml borate-phosphate buffer pH 8.0
2. 40 µg Hg in 0.5 ml borate-phosphate buffer pH 8.0
3. 43 µg Hg in 0.5 ml 0.1 M $NaHCO_3$

be observed. For example, the extraction of amyl-ethyl-barbituric acid and of neopenthyl-allyl-barbituric acid is constant even at high mercury(II) concentrations (up to 200 µg/ml). The distribution ratio of different barbiturates is, however, variable. Barbiturates with long alkylchains, neopenthyl-allyl-barbituric acid for example, are completely extracted after one single treatment, whereas diethyl-barbituric acid is extracted only to 70 per cent (the volumes of the aqueous and chloroform phases being 0.5 and 10 ml respectively).

The determination of mercury in the barbiturate complex

A 1.25×10^{-4} M dithizone solution is used as reagent for determination of mercury (II). To a suitable volume of the filtered chloroform extract, dithizone is added in relation 4 to 1. The determination is carried out indirectly by measuring at 605 mμ, with pure chloroform as reference, the amount of dithizone not consumed in the reaction. The extinction difference between the reagent blank and the sample is a function of the mercury content and consequently of the barbiturate concentration in the sample. Working with the dilution described above, and using a dithizone solution of 32 mg in 1000 ml chloroform, the extinction of the blank in 1 cm cell is about 0.8. This enables the determination of mercury concentrations up to 2 μg/ml. If the concentration of mercury in the sample exceeds this limit, more dithizone and chloroform are added. The actual content will be directly proportional to dilution.

Specificity

The analysis is disturbed by certain drugs, namely hydantoin derivates and 4-ethyl-4-methyl-2.6-dioxopiperidin (Malysol®). These compounds form complexes with mercury(II) similar to barbiturates. Interference by other substances is negligible. The reaction is not disturbed by caffeine, theophyllin, dihydroxypropyltheophyllin, propylthiouracil, sulfonamide derivates, phenacetin, meprobamate, salicylic acid, urea, uric acid, and amino acids. Concerning the specificity of the method, see the publication of Björling *et al.*, 1959. As for the metabolites barbiturates, some preliminary investigatio have been made. If the cyclic structure the metabolite is intact and if only oxidati in the side chain has taken place, it is pr bable that they react with mercury(II however, the solubility and the optimu conditions for extraction can be changed. has been considered to be of interest to stu the behaviour of keto-evipal (cyclohexan-on13-yl)-1.5-dimethyl-barbituric acid[1]. It w found that this compound is extracted ether and forms mercury(II)-complex in t same manner as evipal.

The application of the method for analy of particular barbiturates

For accurate determination of particul barbiturates, a standard curve for ea derivative is necessary. The recovery mercury-barbiturate complexes when extra ting with chloroform varies according to t following table:

5.5-diethyl-barbituric acid	72 per ce
5-phenyl-5-thyl-barvituric acid	90 per ce
5.5-bialløl-barbituric acid	86 per ce
5-isoamyl-5-ethyl-barbituric acid	85 per ce

Because the reproducibility is good af one extraction, no repeated treatment oug to be necessary; and the method will ret its rapidity and simplicity.

The error of the method

The error of the method has been c culated by analysis of 63 serum samples w known concentration of isoamyl-ethyl-b

[1] The authors are indebted to Docent Kurt Skagius who has put keto-evipal at their disposal.

ıric acid added, the concentrations varying
n 0.25 to 2.04 mg/100 ml. In Fig. 2 the
ıdard curve for this compound has been
tted, the lines representing the ± 2 σ
its. A concentration of 0.1 mg per 100 ml
be determined with an accuracy of ± 40
cent, 1 mg per 100 ml with ± 7 per cent,
2 mg per 100 ml with ± 5 per cent. These
iations are calculated for analysis of 1 ml
ım. In cases of barbiturate intoxications,
volume of the sample can be reduced to
ml, and barbiturate concentrations from
to 12.5 mg per 100 ml can be estimated.

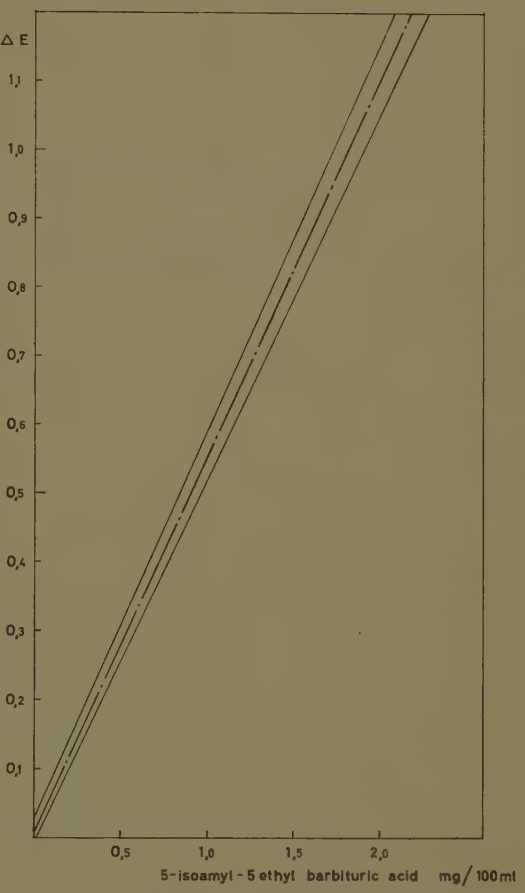


Fig. 2. Calibration curve (± 2 σ) for 5-isoamyl-5-ethyl barbituric acid in serum.

SUMMARY

A micro method for determination of barırates in serum is described. The method ɔased on the extraction of barbiturates n serum with ether, formation of chloron extractable mercury(II) complex in htly alkaline solution, and colorimetric ermination with dithizone of mercury) bound in the complex.

REFERENCES

rling, C. O., Berggren, A., Willman-Johnson, B., Grönwall, A. & Zaar, B.: Determination of barbituric acid derivates as mercury complexes. Acta chem. Scandinav. *12*, 1149, 1958.
rling, C. O., Berggren, A. & Willman-Johnson, B.: Determination of barbituric acid derivates as mercury complexes. J. Pharm. and Pharmacol *11*, 297, 1959.
ıghton, P. M. G.: A rapid ultraviolet spectrophotometric method for the detection, estimation and identification of barbiturates in biological materal. Biochem. J. *63*, 207, 1956.
ing, F.: Estimation of barbiturates in clinical chemistry. Scandinav. J. Clin. & Lab. Investigation *7*, Suppl. 20, 114, 1955.
ry, P.: Action des sels de mercure sur les Veronals. Quelques applications analytiques. J. de pharm. et chim. *2*, 465, 1925.
Gould, T. C. & Hine, C. H.: A modified ultraviolet spectrophotometric method for quantitative determination of barbiturates. J. Lab. & Clin. Med. *34*, 1462, 1949.
Gouldbaum, L. R.: Determination of barbiturates. Ultraviolet spectrophotometric method with differentiation of several barbiturates. Anal. Chem. *24*, 1604, 1952.
Helldorff, I.: On the determination of barbiturates. Scandinav. J. Clin. & Lab. Investigation *7*, Suppl. 20, 127, 1955.

Koppanyi, T., Dille, J. M., Murphy, Wen, S. & Krop, S.: Studies on barbiturates II. Contributions to methods of barbital research. J. Am. Pharm. A. *23,* 1074, 1934.

Levi, L. & Hubley, C. E.: Detection and identification of clinically important barbiturates. Anal. Chem. *28,* 1591, 1956.

Lous, P.: Quantitative determination of barbiturates. A modified spectrophotometric method for clinical use. Acta pharmacol. et toxicol. *6,* 227, 1950.

Pfeil, E. & Goldbach, H. J.: Die Mikrobestimmung der Barbiturate. Ein neues mikrotechnisches Arbeitsverfahren. Ztschr. f. physiol. Chem. *302,* 263, 1955.

Richterich, R.: Die quantitative Bestimmung von Barbitursäure-Derivaten in Körperflüssigkeiten durch ultraviolett-Spektrophotometrie. Clin. Chim. Acta *3,* 183, 1958.

Zwicker, J. J. L.: Recherches et isolement de barbitals, dans les expertises toxicologiques. Pharm. Weekbl. *68,* 975, 1931.

dinav. J. Clin. & Lab. Investigation 231—244. 13. 1961.

FIBRINOLYSIS AND THROMBOPLASTIC ACTIVITY IN RELATION TO HEMORRAGE IN TRANSVESICAL PROSTATECTOMY[1]

By AA. LADEHOFF and J. RASMUSSEN

From the Biological Institute of the Carlsberg Foundation, Copenhagen and the Bispebjerg Hospital, Surgical Department D., Copenhagen

(Received for publication September 30, 1960)

eroperative as well as postoperative norrhage in spite of skilful operative nique is still a problem in prostatic sur- and several ways have been employed recommended for reducing the blood (Goodyear & Beard, 1949), but the hanism of bleeding has not been known ctly. A relation to fibrinolysis, however, ht be possible.

ince detailed reviews of fibrinolytic acti- have been published recently (Astrup, 6, a; Cooper, 1959) only the fundamen- shall be mentioned.

ike the mechanism of blood coagulation, process of fibrinolysis is regulated by a plicated system of activating and in- tory agents. In the organism transfor- ion of the enzyme precursor (plasmino-), which is found in the euglobulin frac- of the blood, into the active protease (plasmin) may take place in two ways, namely a direct and an indirect one. The first of these consists of an interaction between plasminogen and an activator, which can be released for instance from injured tissue. This process is primarily a local one and is considered to play a physiologic role in tissue repair and in the regulation of connective tissue formation (Astrup, 1955). By the second activation mechanism a pro-activator in blood is changed to an activator under the influence of substances with so-called lysokinase effects. This process is general in character and can produce fibrinolysis of variable degrees in the blood.

The degree of plasminemia depends on the relative amounts of active and inhibitory agents. The exact nature and mode of action of the latter are not fully elucidated, but different types are known to exist (Jacobsson, 1955). If the inhibitors are perfectly overcome, all plasminogen may be activated and a fulminant plasminemia, which is the consequence, may result in a break-down of the coagulation mechanism because of dis-

This investigation was supported by grants to Tage Astrup, Copenhagen, from the Josiah y Jr. Foundation, New York, and from the onal Danish Association for Rheumatic Di- es.

integration of plasma fibrinogen, prothrombin and platelets. If not recognized and treated, this state of hemorrhagic diathesis ends fatally in shock.

The often protracted oozing or profuse bleeding from the prostatic fossa in transvesical prostatectomy, and at times the development of severe postoperative shock in spite of adequate replacement of the blood loss by transfusions of whole blood, might suggest local and sometimes also general processes induced by fibrinolytic activity, as local hemostasis is regulated by the balance between clot formation and fibrinolysis (Astrup, 1956, b) and general fibrinolytic activity may interfere with the coagulation ability.

From a surgical point of view it should be important to know if tissue activator, released at the operation site as a result of tissue injury, in addition to local fibrinolysis might cause general fibrinolytic activity in the blood.

Increased fibrinolytic activity in blood has been observed after different surgical procedures (Macfarlane, 1937; Macfarlane & Biggs, 1946; Mathey, Doumet, Soulier, Le Ballogh & Fayet, 1950; von Kaulla & Swan, 1958) and in prostatic surgery after transvesical prostatectomy (Wendt, Perlick & Seyffarth, 1957) as well as in relation to transurethral resection of malignant and hyperplastic prostates (Scott, Matthews, Butterworth & Frommeyer, 1954; Lombardo, 1957), but so far a simultaneous investigation of the fibrinolytic and thromboplastic properties of the enlarged prostate and of the amount of blood loss has not been published.

The purpose of the present paper has been to investigate the fibrinolytic activity blood in relation to transvesical prostate tomy and to estimate the content of plasm nogen activator and thromboplastic materi in hyperplastic prostatic tissue as well as t blood loss, the latter being used as an indic tor of the balance between local clot form tion and fibrinolysis.

MATERIALS AND METHODS

The investigation comprises 25 consecutive pr statectomies in unselected cases of prostatic hype trophy without histologic evidence of malignanc

Operative technique.

The routine method of the department is a on stage transvesical adenomectomy under gener anesthesia. After enucleation of the enlarged pr state arterial hemostasis is secured by ligature the vessels at the posterolateral vesico-prosta junction; packing of the prostatic fossa is m made. A Foley urethral catheter with a 30 ml b loon is placed within the bladder, after which closu of the bladder in two layers with interrupted sutur of catgut and a cystostomy catheter is left at t top of the bladder for 48 hours.

During and after operation the blood loss replaced by transfusions of whole blood and po operative bladder irrigation is performed at inte vals determined by the degree of bleeding.

In the present investigation all operations we done by the chief or associate chief in order secure as uniform and skilful operative technic as possible with a minimum of traumatizing pro dures.

Substrate used for estimation of fibrinolytic activ

The fibrinolytic activity of blood and tissues w estimated by the fibrin plate method (Astrup Müllertz, 1952). Since bovine fibrinogen conta plasminogen, the fibrin is lysed both by plas nogen activators and plasmin, but by heating fibrin plate for 45 minutes at 80° C the plas

en is destroyed (Lassen, 1952) and the fibrin
ts with plasmin only. Thus it is possible to
nguish between activity of activators and of
min.

od samples.

itrated blood samples were taken the day before
ation, some minutes before and after removal
he prostate and after this at different intervals
he per- and postoperative period in order to
blish a possible fluctuation of the fibrinolytic
vity, the last sample being taken the next mor-
.

The samples were immediately spun and the
ma cooled to 0°.C or frozen at —20° C. All
ples from the same patient were assayed in the
e experiment in order that the activities could
compared.

Estimation method. No exact quantitative me-
s yet exist for estimating the single components
he fibrinolytic system in blood separately, but
relative concentrations can be evaluated by
fibrin plate method (Astrup & Rasmussen,
).

The plasma was assayed as whole plasma and
n euglobulin solution obtained after dilution
, precipitation at pH 5.9 (with 1 per cent
ic acid) and redissolution in the original volume.
estimate also the content of proactivator and of
minogen, streptokinase and urine activator were
d to specimens of the euglobulin solution. Serial
ions of the samples and various mixtures were
ared and estimated on normal and heated fibrin.
preparation of the samples before placing on
n plates was made on ice-water bath because
ne marked instability of the fibrinolytic agents
lasma.

ue samples.

or the estimation of fibrinolytic and thrombo-
ic activity, tissue fragments were prepared
the "surgical capsule" adherent to the remo-
prostate, from solitary adenomas and nodular
nternodular tissue. In some cases of diffuse
r fibroadenomatosis a macroscopic separation

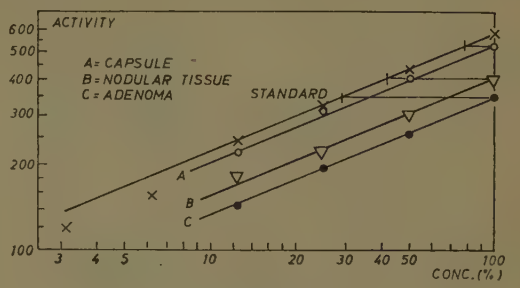


Fig. 1. Estimation of plasminogen activator in a hyperplastic prostate.

Values recorded in a double logarithmic graph. *Abscissa,* concentration of solution in percentage of the stock solution; *ordinate,* fibrinolytic activity recorded as product of two diameters (mm) of the lysed zones on the fibrin plates (triplicates). In the examples *A, B,* and *C* the solutions had respectively 78, 42 and 29 per cent of the concentration of the standard solution.

like this was not possible and a sample of the glandular tissue as a whole was taken. The samples were analysed at once or after storage at —20 C.

Estimation of fibrinolytic activity. The concentration of plasminogen activator was estimated by the quantitative method of Astrup & Albrechtsen (1957). The sample was homogenized and the activator extracted with 2 M KSCN. After precipitation at acid reaction (pH 1 with 1 N HCl) and redissolution, the activities of serial dilutions were estimated on normal fibrin and a dilution curve was recorded (Fig. 1). By interpolation upon a standard curve obtained simultaneously, the concentration of activator was expressed in units of the standard preparation per gram fresh tissue.

Estimation of thromboplastic activity. The content of thromboplastic material was estimated as described by Astrup & Sjölin (1958) and Astrup *et al.* (1959). The tissue sample was homogenized with 0.9 per cent NaCl and the suspension stored at —20 C for a few days. After thawing, retreatment in a homogenizer and filtration through cot-

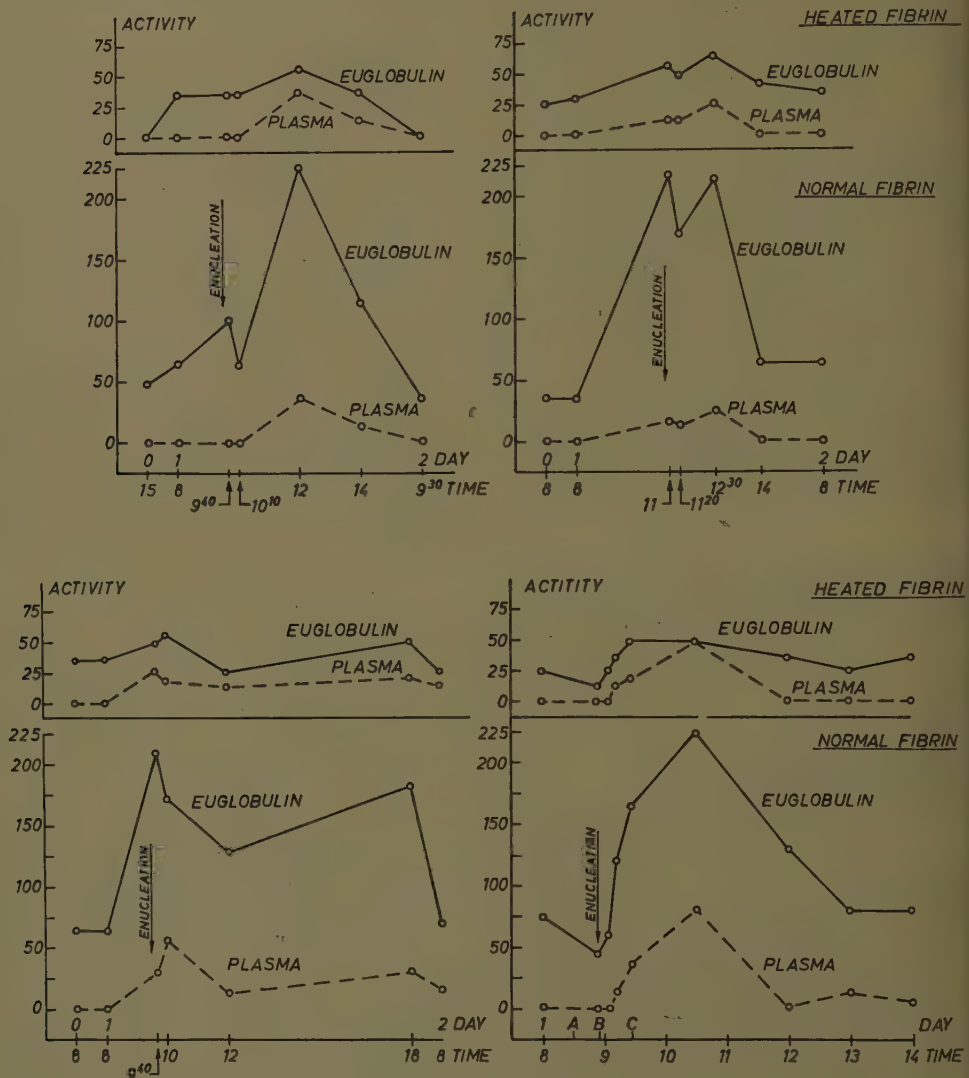


Fig. 2. Fibrinolytic activity of whole plasma and of euglobulin in 4 patients undergoing transvesical prostatectomy.

Abscissa, time for obtaining the blood samples. *Ordinate*, fibrinolytic activity recorded as product of two perpendicular diameters (mm) of the lysed zones on the fibrin plates (triplicates).

In patient IV the time of *A* indicates start of anesthesia, *B* enucleation of prostate and *C* closure of wound finished.

wool, serial dilutions were prepared and the
ing times were estimated by recalcification of
st system with fresh rabbit citrate plasma as a
trate of high spontaneous recalcification time
comparison the activity of a suspension of
an brain was estimated simultaneously and the
mboplastic activity of the tissue sample was
essed as a fraction of this brain dilution curve.

mation of blood loss.

he blood aspirated during operation was meas-
and the gauze serviettes were weighed. The
operative hemorrhage was estimated by spec-
hotometric determination of the hemoglobin
ent in 24-hour specimens of irrigation water
of urine as long as bloodtinge was found.

RESULTS

rinolytic activity in blood.

efore removal of the prostate the activity
low and within normal range and no
uence of anesthesia seemed to appear.
er enucleation, increased activities of both
ma and euglobulin fraction could be
onstrated.

s shown in Fig. 2 the highest activities
hole plasma and of euglobulin on normal
n were found during the first two hours
r enucleation and normal level had been
hed next morning. A similar pattern was
d when plasma and euglobulin were
ed on heated fibrin, which reacts with
min only.

n Fig. 3 the activity of plasma and euglo-
n from another patient is recorded, the
d samples having been taken at shorter
rvals and for a longer period. The highest
ease of activity was reached one hour
enucleation and normal level was not
d until after 8 hours.

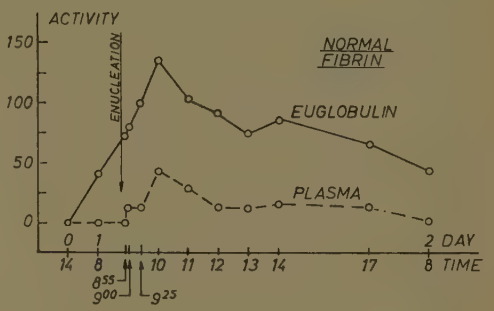


Fig. 3.

Estimations of the activity of euglobulin solutions with streptokinase and urine activator added showed the content of proactivator and of plasminogen to be high in all cases.

In all patients increased activities were demonstrated similar to those mentioned above. Since plasma samples obtained after removal of the prostate lysed both normal and heated fibrin, the effect of the inhibitory agents in blood must have been overcome to a certain extent. This phenomenon, however, was not found in all cases, but only in those with high euglobulin activity.

Fibrinolytic activity of hyperplastic prostatic tissue.

The concentration of plasminogen activator appears in Table I.

The activator content was high in all parts of the prostates, but a considerable difference was found between the activity of the "surgical capsule" and the glandular tissues, as a very high average concentration of 824 units occurred in the former and mean values of 395 and 446 units in the latter. Certainly the activator content in solitary adenomas and nodular or internodular tissue of the

same prostate might differ, but the two mean values were almost identical.

Individual variations of activity could be demonstrated as has been the case in investigations of other human tissues (Albrechtsen, 1957; Ladehoff, 1960).

Thromboplastic activity of hyperplastic prostates.

Examples illustrative of the following are given in Figs. 4 and 5.

As a whole the content of thromboplastic material was large in both capsular ar glandular tissues, but apparently individu variations as well as variations within th different parts of the same prostate (Fig. 4 b) were found. In a few cases (Fig. 4 b, c an activity less than half of that of the bra suspension was demonstrated, in most (Fi 4 d) it was about equal to and in some (Fi 4 e, f) even higher than the latter.

Generally the dilution curves did n parallel the brain curve completely, indica ing qualitative differences. In most cases th

Table I. *Concentration of plasminogen activator in various sections of hyperplastic prostates.*

case	weight (g) of prostate	«surgical capsule»	solitary adenoma	(inter)nodular tissue	gland as a whole
1	48	960	—	—	348
2	40	217	—	—	159
3	33	936	1200	492	—
4	60	624	468	456	—
5	230	936	348	504	—
6	30	—	251	415	—
7	27	696	216	348	—
8	38	480	276	324	—
9	75	552	240	300	—
10	25	1766	398	638	—
11	40	1260	227	328	—
12	25	744	—	—	300
13	30	960	552	240	—
14	25	202	346	78	—
15	35	1440	768	1200	—
16	140	479	308	479	—
17	79	552	372	480	—
18	62	492	312	492	—
19	40	696	204	432	—
20	20	—	—	—	935
21	30	456	108	408	—
22	42	1020	446	586	—
23	25	1500	648	—	—
24	34	1555	219	320	—
25	37	420	—	—	428
Average		824	395	446	434

The figures represent activator units per gram fresh tissue.

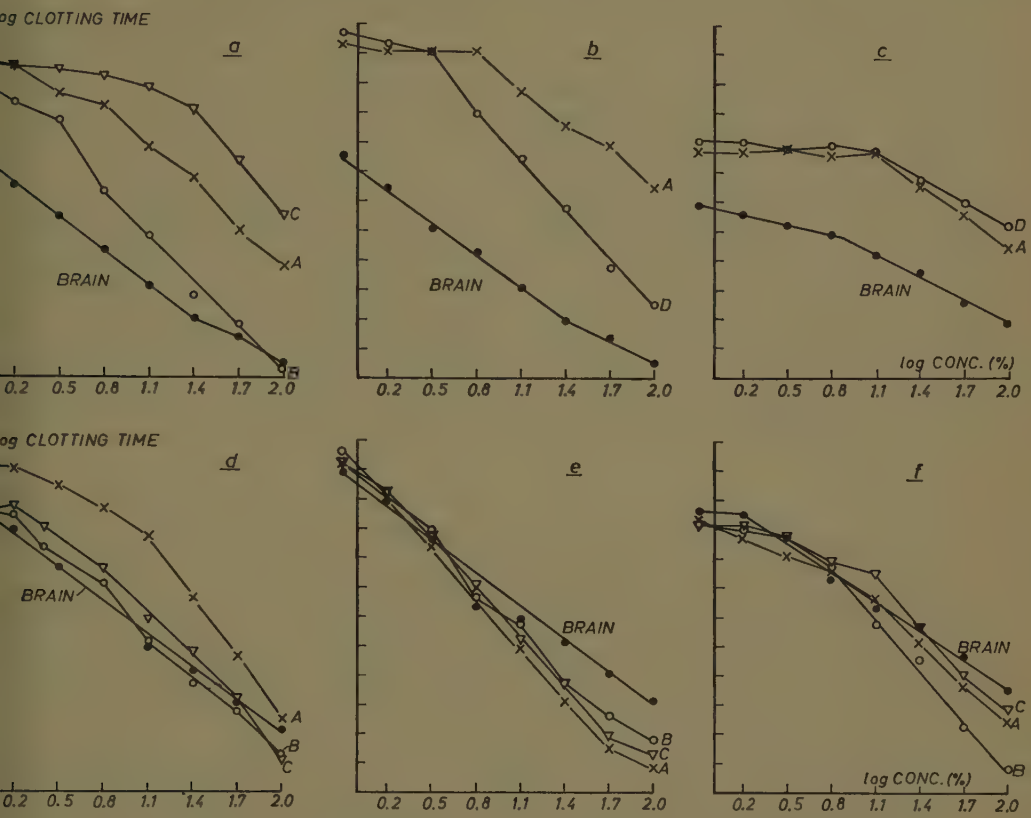


Fig. 4. Estimation of thromboplastic activity in different parts of 6 hyperplastic prostates.

Abscissa, logarithm of concentration in percentage of stock solution. *Ordinate,* logarithm of clotting time (in sec.).

A = "surgical capsule". *B* = (inter)nodular tissue. *D* = glanduar tissue as a whole.

es were somewhat steeper, maybe be- e of inhibitory material in the brain ension, but the dilution curve of the r usually had a straight run in contrast hose of prostatic tissue. Another pro- e explanation of the relatively lower acti- with increasing dilution is that the ing of the substrate plasma may be in- ıced by the high fibrinolytic activity of prostate suspensions because of the lengthening of clotting time conditional on dilution.

In one case (Fig. 5 a) trace of and in three cases (Fig. 5 c, d, e) large amounts of agents inhibiting thromboplastic activity were found. This was also the case of the secretion squeezed from a prostate with large succulent glandular adenomas, while the tissue samples showed no inhibitory effect (Fig. 5 b).

Blood loss in transvesical prostatectomy.

As will appear from Table II, the average total blood loss was 1040 ml, the majority of which took place during operation (579 ml) and the rest of the first day (276 ml). After this time the bleeding was rather slight and usually had stopped after the 6th day.

Two patients (cases 5 and 11) with particularly large blood loss of 3185 and 3957 ml respectively will be reported in detail to illustrate two possible types of bleeding mechanisms.

In case 11 (Table II) the profuse per- and postoperative hemorrhage may be caused a fibrinolytic hemorrhagic diathesis due the prostatectomy, the fibrinolytic activity blood still being high 4 hours after operati (Fig. 6). Moreover this was indicated abnormal bleeding from the perivesical t sue, bladder wound and skin incision aft enucleation. The hemorrhagic state ceas after transfusion of 3500 ml whole blood a local bleeding had stopped after the seco postoperative day. In the prostate the co tent of both plasminogen activator (Table and thromboplastic material (Fig. 4 d) w high.

Table II. *Per- and postoperative blood loss (ml) in 25 transvesical prostatectomies.*

case	during op.	rest of 1st day	postoperative day									total
			2	3	4	5	6	7	8	9	10	
1	171	332	58	15	3	3	—	—	—	—	—	582
2	670	230	21	6	8	8	6	3	4	3	—	959
3	285	52	9	54	1	0	2	1	—	—	—	404
4	422	350	47	12	29	22	7	—	—	—	—	889
5	574	970	748	292	100	39	62	38	204	147	11	3185
6	587	238	49	70	13	9	5	2	—	—	—	973
7	635	180	30	—	—	—	—	—	—	—	—	845
8	407	147	139	47	36	17	14	13	14	14	—	848
9	428	705	140	73	37	23	12	17	13	12	11	1471
10	640	31	7	11	—	—	—	—	—	—	—	689
11	2618	1090	224	17	8	—	—	—	—	—	—	3957
12	496	148	182	98	11	6	7	—	—	—	—	948
13	252	25	21	7	9	7	4	—	—	—	—	325
14	245	120	11	11	4	7	—	—	—	—	—	398
15	682	158	19	11	7	8	9	7	—	—	—	901
16	560	119	70	54	20	28	12	—	—	—	—	863
17	370	485	51	65	21	10	6	11	5	—	—	1024
18	300	218	24	9	3	4	4	—	—	—	—	562
19	587	35	6	7	5	—	—	—	—	—	—	640
20	294	374	24	11	7	15	12	—	—	—	—	737
21	412	21	8	7	10	6	—	—	—	—	—	464
22	990	470	71	166	77	25	19	10	—	—	—	1828
23	400	198	20	30	18	9	—	—	—	—	—	675
24	938	80	72	51	10	15	—	—	—	—	—	1166
25	511	122	25	7	—	—	—	—	—	—	—	665
average	579	276	83	45	17	10	(7)	(4)	(10)	(7)	(1)	1040

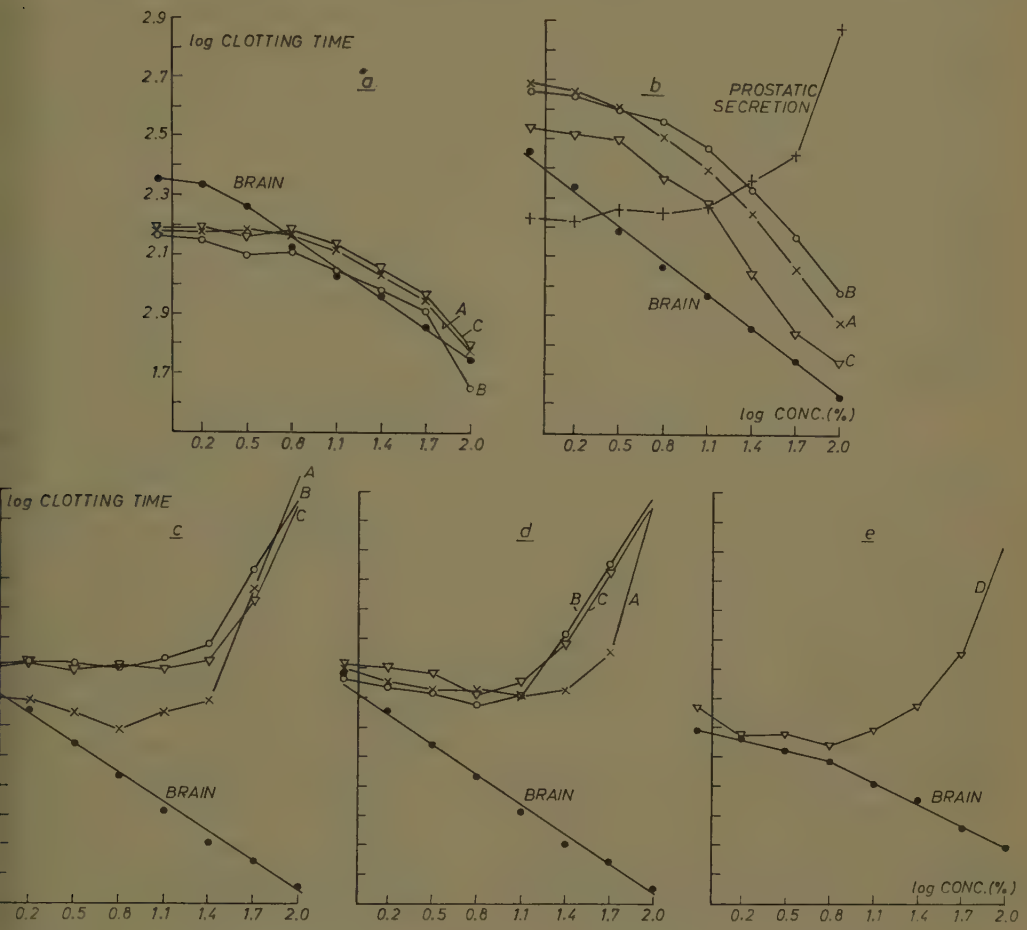


Fig. 5. Estimation of thromboplastic activity in 5 hyperplastic prostates containing inhibitory agents.

Abscissa, ordinate and the letters of A, B, C, D as in Fig. 4.

case 5 (Table I) an adenomatous pro- of 230 g was removed, leaving a large ty with "capsular" tissue of high fibrino- activity (930 units/g) and a thrombo- ic activity of about ¼ of the brain ension (Fig. 5 b). Only a short increase of the fibrinolytic activity in blood was demonstrated. Probably the cause of protracted bleeding (Table II) was mainly local and due to a high fibrinolytic and rather low thromboplastic activity of the walls of a large prostatic fossa.

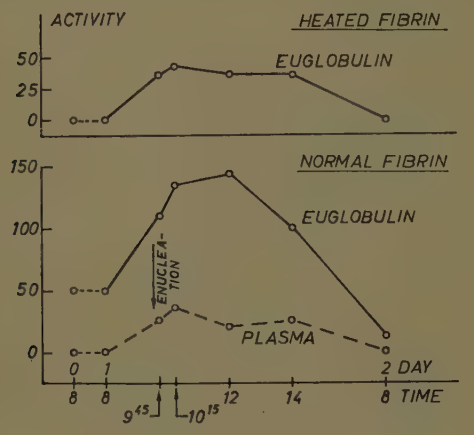


Fig. 6. Abscissa and ordinate as in Fig. 2.

DISCUSSION

A high content of plasminogen activator is found in normal human prostatic tissue (Albrechtsen, 1957) and to some extent the fibrinolytic activity of seminal fluid is caused by a stable plasminogen activator of the tissue type, probably originating from the prostate (Rasmussen, Albrechtsen & Astrup, 1957). The activity is not restricted to the prostate of fertile age, but has also been demonstrated at birth and in early embryonic life (Ladehoff, unpublished investigations).

In tissue cultures of hyperplastic prostatic tissue the clot substrate was lysed by the glandular cells (Röhl, 1959); Källén & Röhl, 1960) and fibrinolytic activity has been demonstrated in extracts of malignant prostates and their metastases (Tagnon *et al.*, 1953). The studies made by Röhl *et al.* are in accordance with the present investigation which showed a very high activator content in the "surgical capsule" and a somewhat lower in the glandular adenomas of hyperplastic prostates in old age. In relation this it should be mentioned that the "su gical capsule" consists of connective tiss and the compressed true prostate, displac peripherally by the hyperplasia of the pe urethral glands.

Several reports on the thromboplas activity of different animal and human t sues are available (Bernheim, 1910; Fisch 1935; Neu, 1909; Reichel, 1942), but far prostatic tissue has not been investigate The present study of hyperplastic prostat showed a generally high, although individ ally variable, thromboplastic activity, but some cases inhibitory agents were found. The individual variability of both fibrinoly and thromboplastic activity found in hyp plastic prostatic tissue may influence t hemostasis in prostatic surgery.

Since part of the plasminogen activator less firmly bound to the tissue proteins (A brechtsen, 1958; Rasmussen *et al.*, 1957) may possibly enter the blood stream und certain conditions and convert plasminog into plasmin, causing increased fibrinoly in the circulating blood.

Besides in non-operative cases of me static cancer of the prostate (Aboulker, S lier & Larrieu 1955; Balfour 1958-59; B gen & Schilling 1958; Crane, Ware & H milton 1955; Dolaz, Dolehide & Friedma 1955; Dossogne 1959; Ledent & De Gee 1959; Marchal, Duhamel, Leroux, Nivet Samama 1959; Miller, Meisel, Jackson Collier 1959; Rasmussen & Schwartz 19 Scott, 1956; Tagnon *et al.* 1952-54) the velopment of fibrinolytic hemorrhagic d thesis has been observed in immediate re tion to transurethral resection of benign p static enlargement in cases with defect

stasis, reported by Scott *et al.* (2 cases) Baurys & Jacobson (13 cases). Five ar cases with hypo- or afribrinogenemia o general fibrinolysis after prostatic re- ons, one of which for cancer, have been ibed by Lombardo (1957) and in a of 30 consecutive resections the same r (1958) found an average drop of 20.3 cent in the plasma fibrinogen level 4 s after operation, but no estimation of olytic activity in the blood was made. ever, a drop in plasma fibrinogen alone proof of increased fibrinolysis when the vascular infusion of appreciable amounts igation fluid during transurethral resec- (Taylor, Maxson, Carter, Bethard & tiss, 1958) is considered.

an investigation to determine whether olled hypotension might lessen the l loss in transvesical prostatectomy, dt, Perlick & Seyffarth, (1957) also ed the fibrinolytic activity in blood, the lysis time method of Macfarlane Independent of the type of anesthesia, ased fibrinolysis appeared in immediate ion to operation, but normal level was l about 3 hours later and even if con- d hypotension seemed to lessen the l loss, estimated by the number of blood fusions given, disastrous postoperative ing might occur. Among 20 cases of atectomy, Ledent & De Geeter (1959) l increased clot lysis of whole blood peratively in two patients and both had ased and prolonged postoperative bleed- During a difficult prostatectomy Fabre, son & Ginoulhac, 1957 observed incoa- ility of the blood shortly after enuclea- of an orange-sized adenoma.

the present study a rise of the fibrino- lytic activity in the blood was observed immediately after enucleation of the prostate and the level remained increased for several hours, but was normal next day. This may partly explain that 80 per cent of the average total blood loss was found within this time, but besides general fibrinolysis the bleeding may be influenced by individual local factors as fibrinolytic and thromboplastic tissue activity, especially of the "capsule", in relation to which the size of the prostatic fossa, that is the amount of removed adenomatous tissue, may play a role.

The general and local factors of bleeding may be illustrated by the two cases (11 and 5) mentioned above, with a blood loss of almost four times the average amount.

Very likely the increased fibrinolysis in blood observed in transvesical prostatectomy is caused by a release of prostatic tissue activator into circulating blood during the manipulations of enucleation together with damage of and absorption from the particularly active "surgical capsule", which is the site of cleavage. In favour of this view may be mentioned the appearance of elevated serum acid phosphatase following massage or surgery of the prostate (Bonner, Homburger & Fishman 1954, Daniel & van Zyl 1952, Dybkær & Jensen 1958, Glenn & Spanel 1959, Hock & Tessier 1949, Marberger, Segal & Flocks 1957, Woodard 1952).

After the lapse of several hours, or at any rate the next day, the normal balance between active and inhibitory agents in blood generally has been re-established and further hemorrhage is influenced by the individually variable fibrinolytic and thromboplastic activity and size of the walls of the prostatic fossa, the border of which consists of dam-

aged bladder mucous membrane with a high content of plasminogen activator as well (Ladehoff, 1960).

In tissue culture studies (Källén & Röhl) a correlation has been found between blood loss and fibrinolytic activity of the glandular cells of the prostate. However, not until a quantitative estimation method of active as well as of inhibitory agents in blood is available may it be possible to correlate fibrinolysis in blood, fibrinolytic and thromboplastic tissue activity and blood loss.

SUMMARY AND CONCLUSIONS

In 25 cases of transvesical prostatectomy, fibrinolysis in blood was estimated before, during and after operation. In all cases increased fibrinolytic activity of several hours' duration was found immediately after enucleation of the prostate.

The hyperplastic prostates were investigated for thromboplastic activity and fibrinolytic activity, estimated as concentration of plasminogen activator. The thromboplastic activity was usually high but varied, and in some cases inhibitory material was observed.

An individually variable but high concentration of plasminogen activator was found in all samples and on an average twice as high in the "surgical capsule" as in the adenomas.

Estimation of per- and postoperative blood loss showed 80 per cent of the total amount to be found within the first 24 hours.

The general and local factors of per- and postoperative bleeding are discussed and illustrated by two cases with particularly high blood loss.

The variable bleeding in transvesical pr statectomy may be related to a rise of gene fibrinolysis in blood, possibly due to a relea of prostatic tissue activator during operatio as well as local fibrinolytic and thromb plastic tissue activity at the operation si Because of increased fibrinolytic activity the blood, regardless of skilful surgery, o must be aware of the risk of fibrinolytic h morrhagic diathesis. Since several quanti tively individual factors, most of which ca not be evaluated beforehand, participate the balance between fibrinolysis and clottin it is not yet possible to predict the degree bleeding, nor if or when a state of hemo hagic diathesis may develop.

REFERENCES

Aboulker, P., Soulier, J. P. & Larrieu, M. Syndrome hémorragique avec fibrinolyse as cié au cancer de la prostate. Presse méd. 353, 1955.

Albrehtsen, O. K.: The fibrinolytic activity human tissues. Brit. J. Haemat. *3*, 284, 19

— The fibrinolytic agents in saline extracts human tissues. Scandinav. J. Clin. & L Investigation *10*, 91, 1958.

Astrup, T.: Fibrinolysis and the formation connective tissues. Third Europ. Rheum Congr., Scheveningen, June 1955. Abstra p. 71.

— Fibrinolysis in the organism. Blood *11*, 1956, a.

— The biological significance of fibrinolysis. L cet *II*, 565, 1956 b.

— & Albrechtsen, O. K.: Estimation of the p minogen activator and the trypsin inhibito animal and human tissues. Scandinav. J. C & Lab. Investigation *9*, 233, 1957.

— Albrechtsen, O. K., Claasen, M. & Rasmus J.: Thromboplastic and fibrinolytic activity

the human aorta. Circulation Research, *7*, 969, 1959.

& Müllertz, S.: The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. & Biophys., *40*, 346, 1952.

& Rasmussen, J.: Estimation of fibrinolytic activity in blood. Proc. 7th Internat. Hematol. Congr., Rome, 1958. Pp. 164—169.

& Sjölin, K.-E.: Thromboplastic and fibrinolytic activity of human synovial membrane and fibrous capsular tissue. Proc. Soc. Exper. Biol. & Med. *97*, 852, 1958.

our, J.: Circulating fibrinolysin in carcinoma of the prostate. Canad. J. Surg. *2*, 138, 1958—59.

ys, W. & Jacobson, H. H. H.: Proteolytic enzymes and prostatectomy. J. Urol. *81*, 676, 1959.

en, S. & Schilling, F. J.: Circulating fibrinolysin in a case of prostatic carcinoma with bony metastases. Ann. Int. Med. *48*, 389, 1958.

heim, B. M.: The relation of the blood-vessel wall to coagulation of the blood. J. A. M. A. *55*, 283, 1910.

s, R., Macfarlane, R. G. & Pilling, J.: Observations on fibrinolysis. Experimental activity produced by exercise or adrenaline. Lancet *I*, 402, 1947.

ner, Ch.D., Homburger, F. & Fishman, W. H.: Some factors other than neoplasms altering the prostatic fraction of acid phosphatase in the serum. Surg., Gynec. & Obst. *99*, 179, 1954.

er, J. Fenimore: The surgical aspects of fibrinolysis. Internat. Abstr. Surg. *108*, 417, 1959.

e, J. J., Ware, A. G. & Hamilton, J.: Spontaneous afibrinogenemia in cancer of the prostate. J. Urol., *73*, 379, 1955.

el, O. & van Zyl, J. J.: Rise of serum-acid-phosphatase level following palpation of the prostate. Lancet *II*, 998, 1952.

z, E. J., Dolehide, R. A. & Friedman, I. A.: Fibrinolysis in patients with metastatic carcinoma of the prostate gland. Illinois M. J. *107*, 142, 1955.

ogne, M.: Le syndrome de fibrinolyse prostatique. Acta urol. Belg. *27*, 145, 1959.

Dybkær, R. & Jensen, G.: Acid serum-phosphatase levels following massage of the prostate. Scandinav. J. Clin. & Lab. Investigation *10*, 349, 1957.

Fabre, P., Boisson, L. & Ginoulhac, C.: Incoagulabilité sanguine au cours d'une adenomectomie prostatique transvésicale. J. d'urol. *63*, *327*, 1957.

Fischer, A.: Die gerinnungsfördernde Wirkung verschiedener homologen Organextrakte. Biochem. Ztschr. *278*, 334, 1935.

Glenn, J. F. & Spanel, D. L.: Serum acid phosphatase and the effect of prostatic massage. J. Urol. *82*, 240, 1959.

Goodyear, W. E. & Beard, D. E.: Blood loss in prostatectomy. J. Urol. *62*, 849, 1949.

Hock, E. & Tessier, R. N.: Elevation of serum acid phosphatase following prostatic massage. J. Urol. *62*, 488, 1949.

Jacobsson, K.: Studies on trypsin and plasmin inhibitors in human blood serum. Scandinav. J. Clin. & Lab. Investigation *7*, Suppl. 14, 57, 1955.

von Kaulla, K. & Swan, H.: Clotting deviation in man during cardiac by-pass: fibrinolysis and circulating anticoagulant. J. Thoracic Surg. *36*, 519, 1938.

Källén, B. & Röhl, L.: The fibrinolytic activity of human hyperplastic prostatae studied in tissue culture. Acta chir. Scandinav. *118*, 240, 1959—1960.

Ladehoff, Aa.: The content of plasminogen activator in the human urinary tract. Scandinav. J. Clin. & Lab. Investigation *12*, 166, 1960.

Lassen, M.: Heat denaturation of plasminogen in the fibrin plate method. Acta physiol. Scandinav. *27*, 371, 1952.

Ledent, J. & De Geeter, L.: Fibrinolyse et hémorrhagie après prostatectomie. Acta urol. Belg. *27*, 151, 1959.

— & De Geeter, L.: Un cas de fibrinolyse prostatique. Acta urol. Belg. *27*, 160, 1959.

Lombardo, L. J.: Fibrinolysis following prostatic surgery. J. Urol. *77*, 289, 1957.

— Studies in the prevention of fibrinolysis occurring in prostatic surgery. J. Urol. *79*, 507, 1958.

Macfarlane, R. G.: Fibrinolysis following operation. Lancet, *I,* 10, 1937.

— & Biggs, R.: Observations on fibrinolysis: spontaneous activity associated with surgical operations, trauma, etc. Lancet *II,* 862, 1946.

Marberger, H., Segal, S. J. & Flocks, R. H.: Change in serum acid phosphatase levels consequent to prostatic manipulation or surgery. J. Urol. *78,* 287, 1957.

Marchal, G., Duhamel, G., Leroux, M., Nivet, M. & Samama, M.: Sur un cas de cancer de la prostate avec metastases osseuses et fibrinolyse. Sang. *30,* 472, 1959.

Mathey, J. J., Doumet, P., Soulier, J. P., LeBallogh, A. G. & Fayet, H.: Hémorrhagies graves au cours d'interventions thoraciques par incoagulabilité sanguine due a la fibrinolyse. Mém. Acad. de chir. *76,* 977, 1950.

Miller, J. M., Meisel, H. J., Jackson, D. A. & Collier, C. S.: Fibrinolysis in a patient with carcinoma of the prostate. J. Urol. *81,* 672, 1959.

Neu, M.: Experimentelles über die Bedeutung der Gerinnungskomponente für den postpartalen Blutstillungsmechanismus. München med. Wchnschr. *56,* 2571, 1909.

Rasmussen, J., Albrethsen, O. K. & Astrup, T.: The fibrinolytic activity in the human prostate and in seminal fluid. Tr. 6th Congr. Europ. Soc. Haemat. *2,* 494, 1957, Copenhagen.

— & Schwartz, M.: Metastatic cancer prostatae with haemorrhagic diathesis caused by fibrinolysis. Ugesk. f. læger, *119,* 1086, 1957.

Reichel, Chr.: Über gerinnungsaktive Substanz aus Placentagewebe. Klin Wchnschr. *21,* 862, 1942.

Röhl, L.: Prostatic hyperplasia and carcinom studied with tissue culture technique. Ac chir. Scandinav. Suppl. 240, 1—88, 1959.

Scott, E. V. Z., Matthews, F., Butterworth, C. & Frommeyer, W. B.: Abnormal plasma pr teolytic activity. Surg., Gynec. & Obst., 9 679, 1954.

— Prostatic enzymes and abnormal bleeding. Urol., *75,* 116, 1956.

Tagnon, H.: Le syndrome de fibrinolyse prost tique. Acta clin. belg., *9,* 99, 1954.

— Schulman, Ph., Whitmore, W. & Leone, L. A Prostatic fibrinolysin. Am. J. Med., *15,* 87 1953.

— Whitmore, W. & Shulman, N. R.: Fibrin lysis in metastatic cancer of the prostate. Ca cer *5,* 9, 1952.

— Whitmore, W., Schulman, Ph. & Kravit S. C.: The significance of fibrinolysis occu ring in patients with metastatic cancer of t prostate. Cancer *6,* 63, 1953.

Taylor, R. O., Maxson, E. S., Carter, F. Bethard, W. F. & Prentiss, R. J.: Volum tric, gravimetric and radioisotopic determin tion of fluid transfer in transurethral prost tectomy. J. Urol. *79,* 490, 1958.

Wendt, H., Perlick, E. & Seyffarth, G. H.: Z Blutung und Nach-blutung bei der Prostate tomie. Arch. klin. Chir., 286, 322, 1957.

Widenbauer, F. & Reichel, Ch.: Über gerinnung aktive Zellsubstanzen. Biochem. Ztschr. 3 100, 1941.

Woodard, H. Q.: Factors leading to elevations serum acid glycerophosphatase. Cancer, *5,* 2 1952.

dinav. J. Clin. & Lab. Investigation 245—248, 13, 1961.

STUDIES ON INTRINSIC FACTOR AND PERNICIOUS ANEMIA

III. INTRINSIC FACTOR ACTIVITY AND B_{12}-BINDING POWER OF ELECTROPHORETIC FRACTIONS OF INTRINSIC FACTOR CONCENTRATES

By H. BERLIN, R. BERLIN, G. BRANTE and S-G. SJÖBERG

From the Research Department, A/B Kabi, Stockholm, the Medical Department, Central County Hospital, Falköping, the Central Laboratory and the Medical Department, Central County Hospital, Eskilstuna, Sweden.

(Received for publication October 5, 1960)

n previous studies (Berlin, Berlin, Bran-Andresen & Sjöberg, 1959) it has been wn that B_{12}-binding power and intrinsic or (IF) activity, determined with the illing technique, were concentrated pro-ionally when a commercial IF concen-e was further purified stepwise about 30-. This seems to indicate that the B_{12}-ling material present in the concentrate osely related to IF.

he purpose of the present study was to plete this study by comparing electro-retic fractions of different IF concen-es with regard to B_{12}-binding power and ıctivity.

MATERIALS AND METHODS

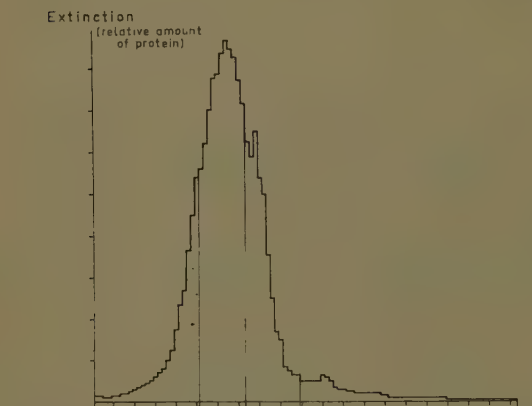
wo IF concentrates were used for the elec-ıoretic studies: 1) The commercial concentrate 98 with a B_{12}-binding capacity of 204 coli (CU) per mg: 2) A further purified material (K 1106) with a B_{12}-binding capacity of 4200 CU/mg and prepared according to the same procedure as described earlier for K 816 (Berlin *et al.*, 1959).

Electrophoresis was carried out on a cellulose powder column. The material was added to the column in a 0.06 M barbital buffer solution, pH 8.6, and eluted with the same kind of buffer. In the eluted fractions (85—115 tubes for each series) relative protein determinations were made according to a modified Folin-Ciocalteau method.

Three series of electrophoresis were made, one on K 798 and two on K 1106.

The eluted fractions were combined to 3—7 larger portions in the different experiments for dialysis to remove barbital, and subsequent determination of B_{12}-binding capacity, expressed in CU, and of IF activity with the Schilling test.

The electrophoretic patterns, the collection of fractions, as well as the content of protein and CU in the different fractions, are given in Fig. 1 for one of the experiments made on concentrate K 1106. In the diagram the mobility towards the anode decreases from left to right. The other two experiments showed essentially the same general picture



Fraction no.	III:1	III:2	III:3	III:4
B_{12}-binding capac., CU/cc	580	14500	4800	60*
CU/mg protein	800	3040	2600	560*
CU total	43000	462000	178000	9000*
Protein, mg	54	152	69	16
Schilling test	Yes	Yes	Yes	Yes

*Assay not very reliable, due to highly diluted solution

Fig. 1. Data for one of the electrophoretic fractionations (Series III) of IF concentrate K 1106 (4200 CU/mg).

and the same results, although the electrophoretic pattern for the less pure material K 798 was considerably more heterogeneous.

In the determination of IF activity in the different fractions, comparisons were made with the same amounts of CU (results from one experiment given in Table I), and in one of the experiments also with the same amounts of protein (Fig. 2).

The methods used for determination of B_{12}-binding capacity and IF activity are described elsewhere (Berlin, Berlin, Brante & Sjöberg, 1958).

RESULTS AND DISCUSSION

It was found, as expected, that the total amount of protein in the different fractions had no correlation to their B_{12}-binding capacity (Fig. 1) nor to their IF activity (Fig. 2).

From Fig. 2 it is also evident that fra tions with a high B_{12}-binding power al had a high IF activity, and fractions witho or with a very low B_{12}-binding power esse tially lacked IF activity.

Table I gives a typical picture of the mo quantitative evaluation of the IF activity different fractions, compared with their B binding capacity.

It is apparent that the IF activity mai accompanies the B_{12}-binding material; ho ever, in all three series electrophoretic fra tions with a lower mobility towards t anode than most of the active material (*e.* fraction III:3 in Table I) tend to show

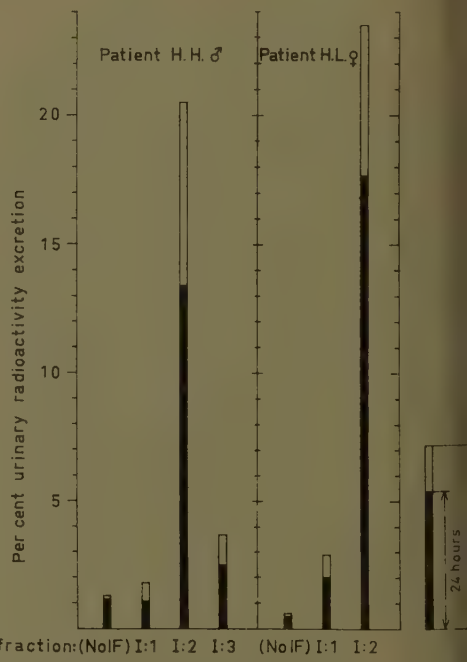


Fig. 2. Schilling test with electrophoretic fracti of IF material (K 798, Series I): Comparison *equal amounts of protein.*

B_{12}-binding capacity of given doses: I:1 < 300 C I:2 3000 CU, I:3 1130 CU.

Table I. *Schilling test (1 µg B_{12}-Co^{60}) with electrophoretic fractions of intrinsic factor material: Comparison of equal amounts of B_{12}-binding material. Values show urinary radioactivity excretion in per cent of given dose. For notation of fractions (III:1 etc.), see Fig. 1.*

	Without IF	IF dose 1000 CU					IF dose 3000 CU					IF dose 1 0000 CU
		Fraction No.				IF Standard	Fraction No.				IF Standard	IF Standard
		III:1	III:2	III:3	III:4		III:1	III:2	III:3	III:4		
G.B. 24 hrs	1.1	10.3	5.5	5.4	8.0	—	22.0	15.6	17.5	22.7	—	28.4
♂ 48 hrs	1.1	13.2	8.1	7.3	10.7	—	28.3	21.5	21.7	28.6	—	34.1
G.A. 24 hrs	1.0	5.1	7.8	4.2	6.3	—	11.3	13.4	9.6	10.6	—	18.1
♀ 48 hrs	1.2	6.9	9.7	5.5	7.8	—	13.4	15.8	12.2	14.3	—	21.6
L.S. 24 hrs	0.5	3.3	5.0	4.3	—	—	10.4	8.7	7.2	—	—	17.1
♀ 48 hrs	0.6	6.5	7.2	6.4	—	—	16.0	13.0	11.2	—	—	24.3
M.O. 24 hrs	0.5	3.1	5.3	4.0	—	7.2	8.6	13.8	8.0	—	8.9	16.8
♀ 48 hrs	0.5	4.7	8.1	6.2	—	9.2	10.3	18.9	11.0	—	11.8	23.5
H.J. 24 hrs	0.2	4.4	3.9	2.8	—	—	11.5	11.7	6.9	—	—	17.2
♀ 48 hrs	0.3	6.1	5.8	4.4	—	—	16.4	17.0	10.9	—	—	23.0
G.L. 24 hrs	—	3.9	3.0	2.5	—	—	9.4	5.6	5.5	—	—	12.0
♀ 48 hrs	—	—	—	—	—	—	—	—	—	—	—	—

ewhat lower IF activity per unit of B_{12}-ing capacity. A definite explanation for cannot be given; one possibility might hat a small amount of either an IF-in-ing factor or an IF-inactive B_{12}-binder mpanies these fractions through the puri-ion stages.

spite of the found irregularity, the ings are compatible with the assumption IF as such does posses B_{12}-binding er.

o our knowledge electrophoretic frac-s of IF concentrates *without* bound nin B_{12} have not earlier been compared B_{12}-binding capacity and IF activity. vever, Gräsbeck (1956) determined IF ity of electrophoretic fractions of hu-gastric juice *with* bound radioactive nin B_{12} and found activity only in the inding fraction. More recently, Barlow rederick (1959) carried out paper elec-trophoresis on hog IF material with radio-active vitamin B_{12} attached to it, and showed that the amount of B_{12}-binding material with a certain mobility, traced through the bound radioactive vitamin, was correlated to the IF activity of the material. On this basis they suggest an *in vitro* assay of IF.

Furthermore, Ellenbogen & Williams (1960) recently were able to prepare a very concentrated IF material with about the same or even higher B_{12}-binding power than our most active fractions and fully active in the Schilling test at a dose as low as 0.3 mg.

These results also support the suggestion that IF activity and B_{12}-binding power are closely related.

SUMMARY

Two different intrinsic factor concentrates from hog's pylorus were fractionated electrophoretically, and the fractions were com-

pared with regard to B_{12}-binding power and intrinsic factor activity, the latter determined with the Schilling technique.

The B_{12}-binding material essentially accompanies the intrinsic factor activity, a fact which suggests a relationship between IF activity and B_{12}-binding power.

Fractions without B_{12}-binding capacity also lack intrinsic factor activity.

ACKNOWLEDGEMENTS

The authors are indebted to the following persons for cooperation in connection with this study and earlier papers in this series: to Dr. N. Nielsen and Mr. B. Holmström, Microbiological Laboratory, A/B Kabi, Stockholm, for carrying out all determinations of B_{12}-binding capacity, to Mr. H. Nihlén and Dr. R. Lundén, LKB Laboratory, Stockholm, for the electrophoretic fractionations, and to Mr. K. Eriksson and Miss A-G. Swärdh, Central Laboratory, Central County Hospital, Eskilstuna, for technical assistance in the radioactivity determinations.

REFERENCES

Barlow, G. H. & Frederick, K. J.: "In vitro ass of hog intrinsic factor concentrates employi paper electrophoresis and Co^{60}–B_{12}." Pr Soc. Exper. Biol. & Med. *101*, 400, 1959.

Berlin, H., Berlin, R., Brante, G. & Sjöberg, S-("Studies on intrinsic factor and pernicio anemia. I. Oral uptake of vitamin B_{12} in p nicious anemia with increasing doses of intrinsic factor concentrate.", Scandinav. Clin. & Lab. Investigation *10*, 278, 1958.

Berlin, H., Berlin, R., Brante, G., Gad Andres J. and Sjöberg, S-G.: "Studies on intrin factor and pernicious anemia. II. Correlati of intrinsic factor activity to B_{12}-binding pow in different hog pylorus preparations.", Sca dinav. J. Clin. & Lab. Investigation *11*, 1 1959.

Ellenbogen, L. and Williams, W. L.: "Prepa tion and properties of purified intrinsic facto Biochem. Biophys. Res. Communications *2*, 3 1960.

Gräsbeck, R.: "Studies on the vitamin B_{12}-bindi principle and other biocolloids of human g tric juice.", Acta med. Scandinav. Suppl. 3 1956.

candinav. J. Clin. & Lab. Investigation 249—263, 13, 1961.

THE EFFECT OF DIFFERENT PATHOLOGICAL CONDITIONS ON THE EXCRETION OF UREA IN THE RABBIT KIDNEY[1]

By M. ANDERSEN

From the Institute of Experimental Medicine, University of Copenhagen, Denmark.

(Received for publication September 30, 1960)

Urea excretion by the mammalian kidney as been the subject of extensive investiga-on, and previously it was the general belief iat this excretion was a passive process.

According to the so-called modern theory f kidney function (Cushney-Rehberg 1926), iere is formed in the glomeruli an ultra-ltrate which contains urea in the same con-entration as in plasma. On its passage irough the kidney this ultrafiltrate is con-entrated, and, following the formation of a oncentration gradient, part of the filtered rea diffuses back into the blood. More ecently investigations by Bodil Schmidt-ielsen and co-workers (1954—57) have iown that an active excretion of urea is emonstrable in mammals. The urea re-osorption percentage is that percentage of ie filtered urea which, during passage irough the kidney, returns to the blood. In 951 this figure was investigated by Effer-ϕe, who found in normal rabbits that the rea reabsorption percentage (Ru) is a logarithmic function of urine flows less than 150 µl/min/kg, and that in a given urine flow the Ru is independent of the filtration, and consequently of the concentration index.

In an earlier work Andersen & Nielsen (1955) confirmed Effersøe's findings and while examining renal function in rabbits during hypothermia, found a considerable variation from the normal. When the temperature was reduced below 33° C the Ru fell to a lower figure than that of normal animals. In the present work, which contains a greater number of normal results, the effect of different pathological conditions on the Ru has been examined. The investigation includes changes in the morphology of the kidney such as compensatory hypertrophy and those caused by partial corticectomy or the action of proteose, and also the effect of functional changes such as caused by hemorrhagic shock, dehydration or the infusion of noradrenaline.

METHODS

The methods used for clearance estimations have been described in the previous study (Andersen & Nielsen 1955). The inulin analysis is carried out

[1] Supported by a grant from "King Christian X's oundation".

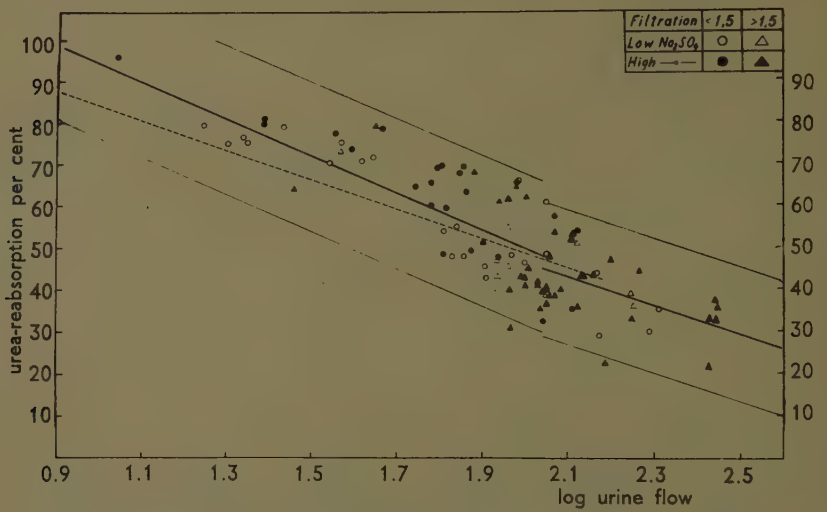


Fig. 1. Normal material consisting of 99 determinations of Ru, plotted against the logarithm of the urine flow The thick continous lines show the regression lines for the Ru determination. The thin continuous lines show the double standard deviation. The dotted line is the regression line for Effersøe's material from 1951. By using different symbols the normal material has been differentiated into high and low filtration rates and high and low sulphate dosages. The regression lines of these results are shown in Fig. 2.

according to the method described by Corcoran & Page (1939) in Bojesen's modification (1949). The urea is determined by Conway's method (1947). In the determination of PAH clearances, the method used for dosage was that of Bergstrand (1952) and for analysis, that of Brun (1951). The diuretic used is Na_2SO_4 in a 20 per cent solution the dose being 0.01 ml/min/kg with the exception of the first 9 animals in the normal material, where the dose was 0.04 ml/min/kg.

NORMAL MATERIAL

The normal material consists of 99 Ru estimations from 18 rabbits. 60 of these 99 estimations are taken from the previous study. In Fig. 1 the relationship of Ru to urine flow is shown. The continuous line is the average curve for all the points, obtai by the method of least squares. The impr sion is that, at the higher levels of urine fl the curve was flatter than at the lower, accordingly the levels above and below µl/min/kg have been considered separat There is, however, no significant differe between the two curves. The dotted lin the average curve drawn from 87 Ru res on 19 rabbits in Effersøe's study on excretion in normal rabbits. The two lines represent the double standard deviat which, according to definition, must incl 95 per cent of the points.

Fig. 2 shows the curves for the nor material, calculated for Ru determinat

ring high and low filtrations. There is no gnificant variation between these two cur-s. In the same figure are shown the curves hich have been calculated for Ru deter-nations during high and low sulphate sage, and again there is no significant riation in the two curves.

Statistics. The equation for the regression line the normal material for urine flows less than) μl/min/kg is Ru = 138.99− 44.63 x log. urine w. With urine flows greater than 110 μl/min/kg e equation is Ru = 114.98− 34.28 x log. urine w. Comparing the two curves one finds that the b values (the slope of the curve) t = 0.94, d for the y values (the height of the curve over e base line) t = 1.16. That is, there is no signifi-nt difference between the two curves.

The regression line for Ru estimations at high rations is expressed by the equation Ru = 131.69−-06 x log. urine flow, and at low filtrations by e equation Ru = 136.89− 42.93 x log. urine flow. ter identity testing it was found that in the b lues t = 0.12 and in the y values t = 1.74 indi-ing that there is no significant difference between e two curves. The regression lines for Ru deter-nations for both high and low sulphate dosage n be expressed by the equations Ru = 152.68− 40 x log. urine flow, and Ru = 162.28− 41.37 x g. urine flow respectively. Comparing the two rves it is found that for the b values t = 1,31 d for the y values t = 1.31. That is, there is ain no significant difference between the two rves.

Discussion. The above investigation con-ms Effersøe's findings: 1) Within the nge of examination Ru is a logarithmic nction of urine flow. 2) At a given urine w Ru is independent of the filtration rate, d consequently of the concentration index. Variation in the dose of sodium sulphate es not appear to alter the relationship be-een urine flow and Ru. In common with hers the author demonstrated a great

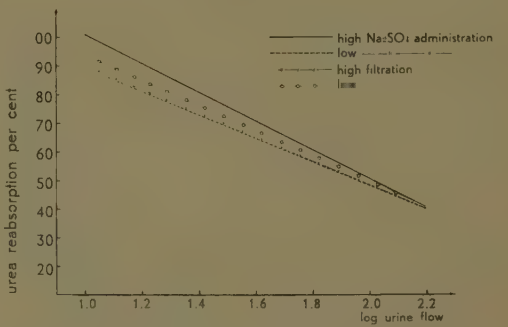


Fig. 2. The regression lines for the different groups of Ru estimations shown in Fig. 1. There is no significant variation between the regression lines for determinations at high or low filtration, and again no significant variation between the regression lines for Ru determinations at high or low sulphate dosages.

scattering in the results, especially in the range of values bordering upon 100 µl/min/kg.

The curve drawn from Effersøe's results is, for the lesser urine flows, a little flatter than that drawn from the author's material, whereas it is parallel to the curve for the higher urine flows. This is probably due to the fact that Effersøe's curve incorporates relatively more results for the higher urine flows.

A. THE EFFECT OF CHANGES IN THE MORPHOLOGY OF THE KIDNEY

I. *Compensatory Renal Hypertrophy*

It has been shown by Oliver (1950) that, in the case of compensatory renal hypertrophy, the predominant part of the hypertrophy falls on the proximal tubules, where an increase of more than 100 per cent in the diffusion area occurs. In the remainder of the nephron and in the collecting tubules any increase is minimal. According to Homer

Table I. *The calculation of the relative hypertrophy of the kidney has been made in the following way. An allowance was made for the growth of the animal after uninephrectomy and an estimation made of the possible weight of the removed kidney at the time of clearance experiment. This estimated weight was compared with the weight of the hypertrophied kidney. Within the range 90 til 170 days following uninephrectomy, there was found to be no relationship between the relative hypertrophy and time after unilateral nephrectomy. In the calculation it has been assumed that the original weight of the two kidneys was the same (Addis et al. 1924).*

Rabbit no.	Days between uninephrect. and clearance estimations	Weight of rabbit at the time of opr. and at the day of clearance estimation	Weight of kidney removed and of the hypertrophied one.	Relative hypertrophy in %
		kg.	g.	
I.	90	1. 2,45 2. 2,45	1. 7,1 2. 14,6	105,5
II.	120	1. 2,40 2. 2,00	1. 5,8 2. 9,8	102,5
III.	120	1. 2,10 2. 2,30	1. 6,0 2. 9,5	44,5
IV.	120	1. 2,10 2. 2,45	1. 6,8 2. 12,6	58,7
V.	135	1. 2,10 2. 2,60	1. 5,8 2. 10,5	46,4
VI.	140	1. 2,00 2. 2,05	1. 6,7 2. 9,9	44,2
VII.	160	1. 2,46 2. 2,60	1. 6,4 2. 11,8	74,6
VIII.	170	1. 2,00 2. 2,20	1. 6,5 2. 9,7	35,8
IX.	90	1. 2,80 2. 2,95	1. 7,5 2. 13,6	72,0
X.	90	1. 2,40 2. 2,50	1. 6,8 2. 13,8	95,0
XI.	94	1. 2,00 2. 2,40	1. 5,7 2. 10,2	47,0
XII.	101	1. 2,50 2. 3,15	1. 7,8 2. 14,5	46,5
XIII.	118	1. 2,25 2. 2,85	1. 7,0 2. 14,5	63,5

Smith (1951), the major part of the wa content of the glomerular filtrate is absorbed in the proximal tubules. It is be expected, therefore, that the urea absorption in the hypertrophic kidney diff from the normal. In order to find out if s is the case, the following experiment carried out.Thirteen rabbits of weights tween 2 and 3 kg were used. Unilateral phrectomy was carried out under ureth anesthesia, and clearance estimations formed between 90 and 170 days after operations. The weight hypertrophy of remaining kidney ranged between 36 and per cent (average 64 per cent). It was ther shown that, within the above mentio space of time, there was no relationship tween the increase in the renal wei and the time elapsing after nephrecto (Table I).

Fig. 3 shows the result of 88 Ru de minations on rabbits with compensat renal hypertrophy. The regression lines double standard deviation for the nor material are shown as dotted lines and t for the animals with compensatory r hypertrophy as continuous lines. In the s way as in the previous experiment a dist tion was made in the plottings as to whe the urine flows were above or below 110 min/kg. It was found that with urine fl both above and below 110 µl/min/kg t was no significant difference in the regr ion lines for the hypertrophied kidneys. A with urine flows below 110 µl/min/kg, regression lines representing the normal the hypertrophied material showed no si ficant difference. It was further found in urine flows above 110 µl/min/kg, t was no significant variation in the slope

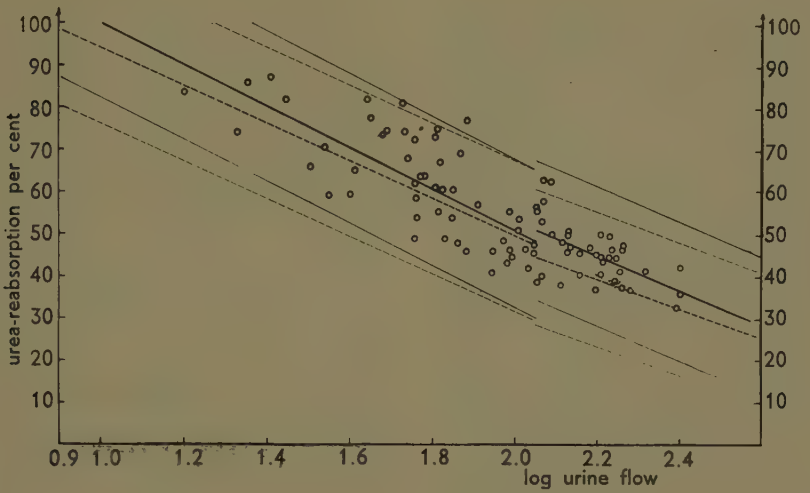


Fig. 3. The figure shows 88 Ru determinations from 13 animals with compensatory renal hypertrophy. The continuous lines show the regression lines and double standard deviation for this material. The dotted lines show the regression lines and double standard deviation for the normal material.

curves. On the other hand, however, the ve for the hypertrophied organs lay signi- antly higher thant that of the normal.

Statistics. The regression line for Ru determi- ions in urine flows below 110 μl/min/kg has equation Ru = 150.45− 49.87 x log. urine flow. e equation for urine flows above 110 μl/min/kg Ru = 134.43− 40.69 x log. urine flow. Identity ing of these two curves gives for the b values 0.65 and for the y values with a given x, 0.96.

In comparison with the normal material in urine ws below 110 μl/min/kg it is found that for the values t = 0.66 and for the y values t = 1.3 the curves representing urine flows greater n 110 μl/min/kg it is found that for the b values 0.39 and for the y values t = 3.00.

Discussion. As regards the larger urine ws, there has thus been established a small, but significant variation from the normal. Although this difference exists, practically all the plottings from the hypertrophic kidneys fall within the normal range of variation. The explanation of the deviation presumably is that the influence of proximal water reabsorption is brought to bear particularly in the larger urine flows. Considering the very pronounced changes in the morphology of the kidneys, the functional deviation is in any case small.

II. *Partially Conticectomised Kidneys.*

It has been particularly emphasised by Trueta, Barclay, Daniel, Franklin & Prichard (1948) that two types of nephrons are found in the kidney, namely, the juxtamedullary comprising 15 per cent of the total (Heggie 1947), and the cortical. The essen-

tial difference between these two types, apart from the length of tubules, lies in the structure of the blood supply. The efferent vessels from the cortical glomeruli subsequently split up into a capillary network which surrounds the tubules. The efferent vessels from the juxtamedullary glomeruli, on the other hand, proceed directly into the medulla as *vasa recta,* coming particularly in contact with the loops of Henle and the collecting tubules. Bing (1949), employing a special technique, has shown that it is possible to remove a large part of the kidney cortex without causing the death of the animal from hemorrhage. The same author has also worked out an onchometric method of determining the portion of tissue removed as a percentage of the renal volume (1955).

In a recent experiment 3 out of 7 partially renal corticectomised rabbits were kept al after removal of between 23 and 38 per c (average 33 per cent) by volume of e kidney. It was attempted to form a rou estimate of the residual functioning nephr by counting the intact glomeruli in vari sections and comparing with similar secti from normal kidneys. In 20 areas there w thus counted 108 in the corticectomised a 232 functioning glomeruli in the nor kidneys.

After about 2—3 months, when the a mals had completely recovered from operation, clearance estimations were ma The results of these estimations, in all 40 determinations, are given in Fig. 4.

As previously, the regression lines a double standard deviations for the nor material are shown as dotted lines.

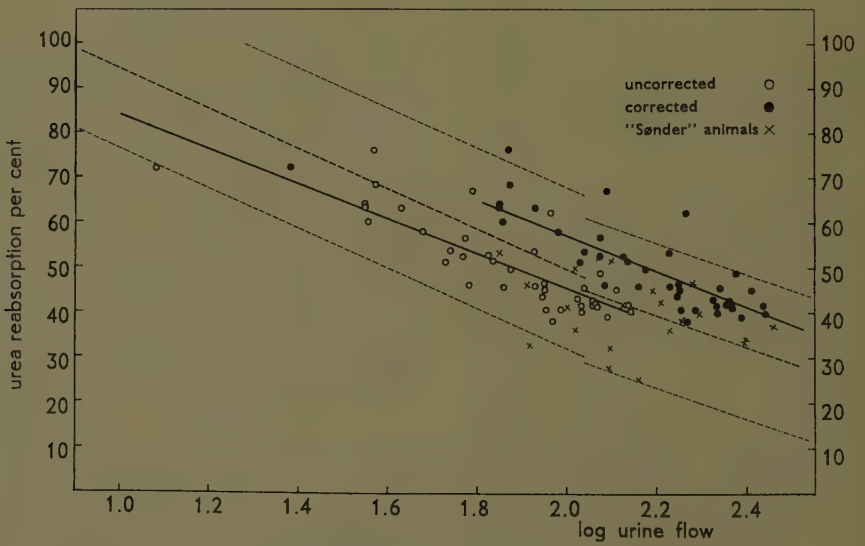


Fig. 4. 40 Ru determinations from 3 animals subjected to bilateral partial renal corticectomy. The lower continuous line is the regression line for the uncorrected results, the upper continuous line is the regression line for the corrected results. The dotted lines have the same meaning as in Fig. 3.

rves for the corticectomised kidneys are own as continuous lines, the lower for the corrected values, the upper for the corted. These corrections are made because, en half of the nephrons are removed from kidney, those remaining are required to oduce twice the urine flow each, to get the ne total diuresis as the intact kidney.

It can be observed that the slopes of the rves for the corticectomised kidneys vary le from the normal, whereas there is a tinct difference in their position on the aph. This latter difference is statistically nificant, the curve for the uncorrected ues being lower than the normal, while t for the corrected values is higher.

In the same figure there are shown, in dition, the Ru values from 21 clearance imations on 4 rabbits used by Sønder in work on experimental hypertension 953). These animals were unilaterally phrectomised and thereafter made hypersive by the application of a Goldblatt mp to the remaining renal artery. They re finally made normotensive again by rtial renal corticectomy. The figures obned are uncorrected, as the portion of the rtex removed was not measured. However, was smaller than in the above experiments. most all the values obtained lay within the uble standard deviation. The regression e produced is very flat, and the slope fers significantly from the normal. On the ner hand, there is no difference in the ight of the curves on the graph.

Statistics. The equation for the regression line the uncorrected values is Ru = 122.84− 38.79 og. urine flow. In comparison with the normal terial it is found that for the b values t = 0.97 l for the y values t = 3.80. The equation for the corrected values is Ru = 134.51− 38.79 x log. urine flow. In comparison with the normal material it is found that for the b values t = 0.44 and for the y values t = 6.29. The uncorrected figures are compared with the normal curve for urine flows below 110 μl/min/kg. The corrected figures are compared with the normal curve for urine flows greater than 110 μl/min/kg. The curve for those animals used in Sønders work has the equation Ru = 73.47− 15.63 x log. urine flow. In comparison with the normal curve it is found that for the b values t = 4.13 and for the y values t = 0.17.

Discussion. It would be anticipated that following partial corticectomy, where a conciderable portion of the long nephrons with their vigorous blood supply is removed, a reduction of urea reabsorption would occur. However, in the curve for the corrected values, it appears that there is an increase in the percentage of urea reabsorption compared with the normal material. This phenomenon can presumably be explained by the increase in permeability, probably in the walls of the tubules and possibly also in the loops of Henle and the collecting tubules, which occurs in a seriously damaged kidney. The variation in the Ru values from the normal is still rather small, and the investigation of renal function in the rabbits used by Sønder shows that even severe interference, such as lowering the blood supply followed by partial corticectomy, does not result in any essential alteration in the urea reabsorption levels.

III. *Treatment with Proteose*[1].

Bing and co-workers (1946, 1951) have described the changes in the renal tubules in

[1] Grateful acknowledgement is made to the Leo factory for providing the hyaluronidase used in the experiment, and to the Ferrosan factory for providing the protein hydrolysate used.

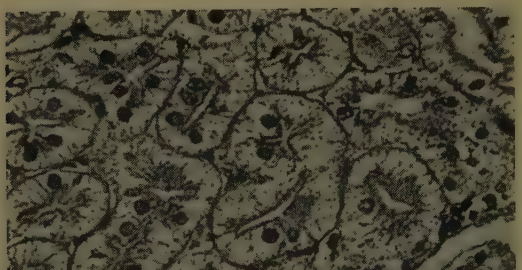


Fig. 5. Microphotograph from a section of kidney from a proteose treated rabbit. The lumen of the tubules is reduced and the epithelium is swollen. The cytoplasm is pale and finely granular. Some of the nuclei are only lightly stained. The changes resemble, but are not identical with, those following the injection of hypertonic sucrose. (Description signed K. Schourup.)

rats produced by the intraperitoneal injection of a protein hydrolysate, the histological picture resembling that found in the so-called "Nephrotic Syndrome". An attempt was made by the author to treat rabbits in a similar way, but without success, as the animals quickly succumbed from peritonitis. Intravenous injection of the hydrolysate was similarly impracticable on account of venous thrombosis or collapse of the animal. The difficulty was overcome by the subcutaneous injection of 3 units of hyaluronidase immediately prior to the injection of the hydrolysate through the same cannula. In this way the hydrolysate was resorbed from the subcutaneous tissue. Employing this method it was possible to produce the characteristic changes in the renal tubules of the rabbit, less pronounced, however, than in the rat Fig. 5. It was also possible to make clearance estimations on two rabbits which had received 1250 and 2055 g of proteose soluti in the course of 14 and 20 days respective The weights of these rabbits prior to the jections were 2950 and 2100 g. At the ti of the clearance estimations the weights w 2550 and 2110 g. The urine showed p nounced proteinuria and numerous granu casts.

The results of the clearance estimatio are shown in Fig. 6. All the Ru estimatio are above the normal curve, but within double standard deviation. There is sig ficant difference in the height above the b line between the normal curve and the gression line for the proteose treated a mals, but there is no significant difference the slope of the two curves.

Statistics. The regression line for the prote treated animals has the equation Ru = 146.2 45.18 x log. urine flow. Identity testing with normal material gives for the b values t = and for the y values t = 2.52.

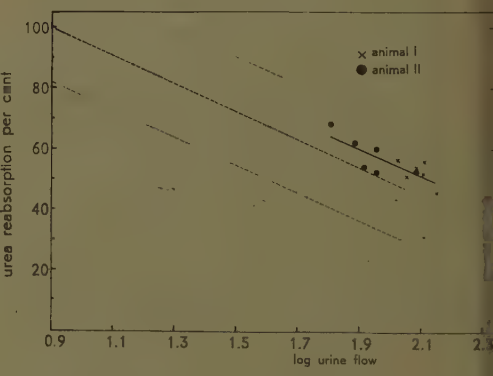


Fig. 6. 12 Ru determinations from 2 animals tre with protein hydrolysate. The regression through these points (continuous) differs sig cantly from the regression line of the nor material (dotted).

Discussion. Effersøe in 1951, making determinations of the Ru in rabbits poisoned with sodium benzoate, found values which suggested an increase of urea reabsorption. There was no demonstrable alteration in the renal histological picture. In the proteose treated animals rather pronounced changes were found in the renal tubules, resembling those found in the "Nephrotic Syndrome" in humans. Bing & Bjering (1937) have found in isolated cases that, in patients with this syndrome, there is a marked variation from the normal relationship between filtration rate and urea clearance. A significant, but smaller variation from the normal is found in the author's investigation. The probable explanation of the increased reabsorption in the kidneys of the proteose treated rabbits lies in the damaged tubular walls.

B. THE EFFECT OF FUNCTIONAL INFLUENCES ON THE KIDNEYS

I. *Dehydration.*

The action of the anti-diuretic hormone (ADH) is believed to be an increase of the water reabsorption in the distal tubules, and in the dehydrated animal one would expect the production of this hormone to be maximal. Should the obligatory proximal water reabsorption be partially abolished by the administration of an osmotic diuretic to a dehydrated animal, one would expect that the major part of water reabsorption would be distally displaced in the tubules.

Two rabbits were deprived of water for three days and given only corn and hay. The influence of dehydration on the Ru was then investigated. The hemoglobin levels increased from about 90 to about 110 per cent and

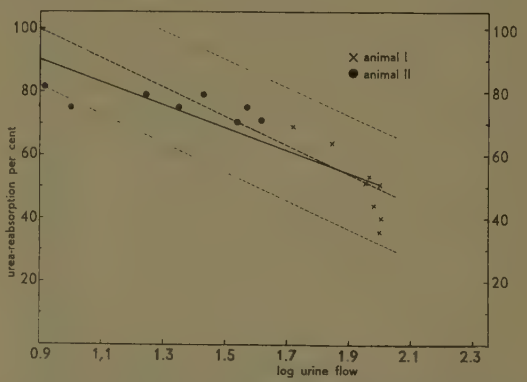


Fig. 7. 16 Ru determinations from 2 dehydrated animals. The regression line for this material does not differ significantly from that of the normal material.

the weight fell about 300 g (3150 to 2820 g and 3400 to 3100 g). Fig. 7 shows the results of the Ru estimations. The regression line for these estimations shows no significant variation from that of the normal material.

Statistics. The regression line for the dehydrated animals has the equation $Ru = 121.34 - 35.61 \times \log$. urine flow. Compared with the normal material it is found for the b values that $t = 1.22$ and for the y values $t = 0.11$.

Discussion. It has been shown that no significant change in the urea reabsorption occurs in the dehydrated animal after administration of an osmotic diuretic. If, as believed, a distal displacement of the water reabsorption takes place, it would be expected that the Ru values would be reduced due to the decrease of the time available for the higher diffusion gradient to exert its influence.

In the experiment with the hypertrophic

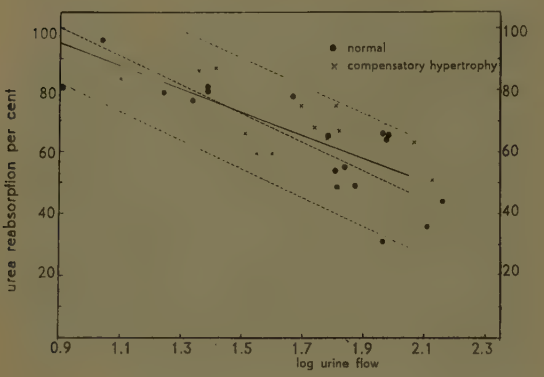


Fig. 8. All the final determinations from 31 clearance experiments both in normal animals and those with compensatory renal hypertrophy. At the time when those determinations were made, the rabbits were hypotensive and probably shocked. There is no significant difference between the regression line of this material and that of the normal.

kidneys, where an increased water reabsorption in the proximal tubules was anticipated, only a minimal alteration in the Ru took place. Taken together these two findings might suggest that a considerable part of the urea reabsorption takes place distal to the nephron, that is in the collecting tubules. Such has also been suggested by Effersøe (1951) and the investigations of Ullrich & co-workers (1958), who cannulised the collecting tubules and examined urine samples from different levels, again seem to confirm this theory.

II. *Hemorrhagic Shock.*

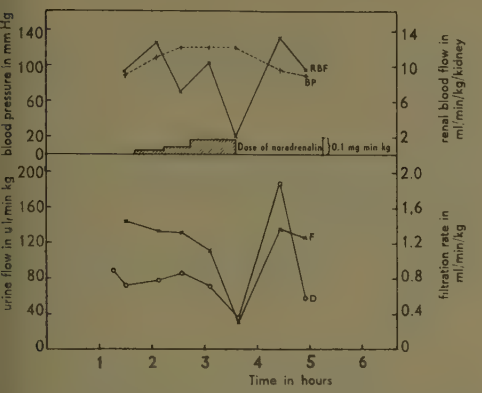
In 1948 Trueta and co-workers showed that it is possible to alter the distribution of the renal blood circulation. By inducing hemorrhagic shock they caused pallor of the kidneys, indicating cortical ischemia. Cortical pallor was observed towards the end of the

clearance experiments in this investigati
where the animals were in a state of sh
resulting mainly from the blood loss to
estimations. The blood pressure fell betw
20 and 50 mm Hg. In two of the experime
on normal and in five rabbits with comp
satory hypertrophy the pale kidney was
posed. It was observed that renal funct
could continue when the blood pressure
as low as 30—40 mm Hg.

In order to examine the effect of hem
hagic shock on the Ru, the final estimati
have been taken from the normal and
hypertrophic material. The figures are s
in Fig. 8. Practically all these values
within the normal range of variation,
there is no significant difference between
regression line of the final estimations
that of the normal material.

Statistics. The regression line for the f
determinations has the equation Ru = 127.
36.51 x log. urine flow. In comparison with
normal material it is found that for the b va
t = 1.14 and for the y values t = 1.05.

Discussion. As was believed to be the
in the corticectomised kidney, it is thou
that the greater part of the urine produc
in the pale kidney takes place in the ju
medullary glomeruli. While hemorrh
shock caused no demonstrable effect on
Ru levels, renal corticectomy appeared
cause significant variation from the nor
but this variation was in a direction oppo
the one expected from theoretical consid
tions. This difference in behavior might
suggest that the effect of corticectomy on
Ru was due to damage to the tubular
and not to the loss of a number of cor
glomeruli.



7. 9. The figure shows blood pressure (BP), al blood flow (RBF), filtration rate (F) and ne flow (D) in the course of an experiment in ich noradrenaline was infused in increasing es. The picture is rather similar to that seen an experiment with progressive lowering of the dy temperature (Andersen and Nielsen 1955).

III. *Noradrenaline Infusion.*

Andersen & Nielsen (1955) demonstrated at, under artificial hypothermia, the kidney comes pale, the urine flow decreases and e Ru falls below normal, this fall becoming eater with the fall in temperature. They ggested that these changes could be the sult of a humorally produced vasoconstric- n in the kidney, as decreased renal blood w and pallor of the kidney have been pro- ced by Moyer, Conn, Markley & Schmidt 949) using injections of adrenaline.

In order to investigate this, the effect of radrenaline infusion on renal function was served.

A preliminary investigation was carried t in which the left renal vein was cannul- d and the renal blood flow directly meas- ed using the method of Barcroft & Brodie 905). The dose of noradrenaline which luced the blood flow by half was deter- mined and found to be 0.0035 mg/min/kg.

Fig. 9 shows an experiment with increas- ing noradrenalin dosage. A comparison of this experiment and a previous one (Ander- sen & Nielsen 1955), in which the effect of steadily decreasing temperature on renal function was studied, indicated that, as re- gards urine flow and filtration rate, there was a marked similarity.

The relationship of Ru to urine flow in experiments with noradrenaline is given in Fig. 10. At the lowest levels of dosage almost all the points lay within the normal variation. The curve through these points is somewhat flatter than the normal, but there is no significant variation in the height over the base line of the two curves. At the higher dosages it can be seen that the Ru values are lower. The regression line for these values is significantly lower than the normal curve. At the highest dosages most of the plottings are far below the limit of the normal varia- tion, and they are so scattered that any curve through them would be valueless.

In Fig. 11 there are seen, as a control, determinations of the Ru prior to and fol- lowing the infusion of noradrenaline. The curve through these points is practically identical with the normal.

The relationship between the renal blood flow estimated by the PAH clearances, and that directly measured by using the Bar- croft-Brodie technique, is shown in Fig. 12. Eight determinations from two rabbits which recieved no noradrenaline are shown to the left of the figure. The calculated blood flow is between 66 and 150 per cent (average 106 per cent) of that directly measured. Seven estimations from three rabbits during infu- sion of noradrenaline are shown to the right

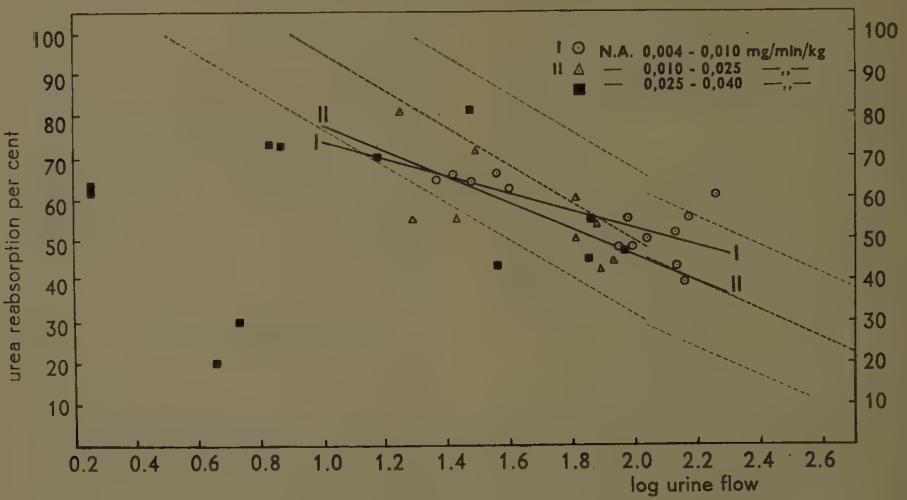


Fig. 10. The relationships of the Ru in rabbits under the influence of noradrenaline. The regression line for the Ru determinations at low noradrenaline dosage, marked I, does not differ significantly from that of the normal material (dotted) in height over the base line. The regression line for the Ru determinations at a somewhat higher noradrenaline dosage marked II, lies significantly lower in the graph than that of the normal. Because of the very great scatter of points, no regression line has been drawn for the Ru determinations at the highest noradrenaline dosages. Most of the points lie far below the normal.

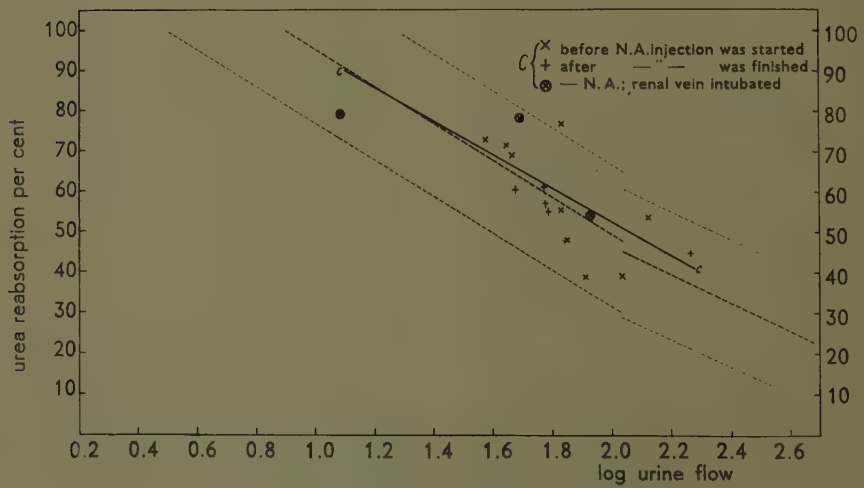


Fig. 11. The control values taken from the noradrenaline experiments. These determinations were made either before or after the infusion of noradrenaline. The regression line for those determinations is practically identical with the normal.

the figure. The calculated blood flow in ery case is far below that measured, being tween 17 and 53 per cent (average 34 per nt) of the measured figure. Increase of the se of noradrenaline apparently results in a eater variation between the calculated and easured renal blood flow. Study & Skipley 949), when stimulating renal nerves, ob-rved a similar phenomenon.

Statistics. For the estimations with the lowest radrenaline dosage the equation is Ru = 94.27− 73 x log. urine flow. Comparing this with the rmal curve, for the b values t = 3.43 and for y values t = 0.55. For the somewhat higher radrenaline dosages the equation is Ru = 108.71− 45 x log. urine flow. Comparing this with the rmal, it is found that for the b values t = 1.01 d for the y values t = 2.24. A comparison of the ves for the lowest and the somewhat higher adrenaline dosage gives for the b values t = 0.79 d for the y values t = 0.35.

Discussion. It can be seen that a consider-le similarity exists in the reaction of the bbit kidney to the influence of hypothermia d noradrenaline, both causing a fall in ine flow and filtration rate. There is also considerable decrease in the Ru values at e lowest temperatures and at the highest radrenalin dosages. Taking into account e pronounced renal pallor found in both ses, it could be assumed that the blood w in a considerable part of the cortex is eatly reduced, and consequently that the mber of functioning nephrons has been de-eased. This satisfactorily explains the de-ease in filtration rate and urine flow, and o explains the decrease in the Ru figures the high noradrenalin doses or low tempe-tures. Should a reduction of, say 90 per nt, in the number of functioning nephrons effected, it would be necessary for the re-

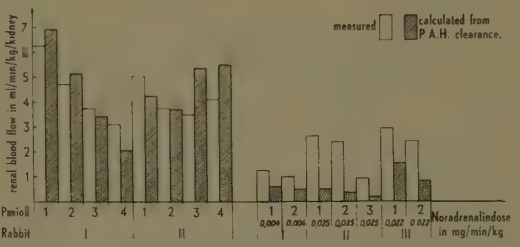


Fig. 12. A comparison between the directly measured renal blood flow and that estimated from the PAH clearance. The 8 columns to the left of the figure show the values from experiments in which no noradrenaline was given. The 6 columns to the right of the figure show the values obtained during noradrenaline infusion. There is a large and constant variation between the measured and the estimated renal blood flow in the noradrenaline experiments.

maining 10 per cent to increase their output tenfold in order to yield the same total output as the normal kidney. For example, the Ru values observed at a urine flow of 10 µl/min/kg, in reality corresponds to a urine flow of 100 µl/min/kg, in which case the Ru values are considerably lower.

A similar effect was not observed in hemorrhagic shock in spite of the fact that the kidney was pale. The explanation could be that the ischemic glomeruli were so few in number that their effect on the Ru was submerged in the scatter of the values. The great difference observed between the measured and the calculated renal blood flow during infusion of noradrenaline would seem to confirm the presence of an A-V shunt mechanism in the kidneys as postulated by Trueta and co-workers. Even if a considerable proportion of the nephrons were not functioning, it would be expected, provided that normal function continued in the remaining nephrons, that the PAH clearance would correspond to the blood flow.

SUMMARY

The Urea Reabsorption Percentage (Ru) may be defined as that percentage of the filtered urea which, during passage through the kidney, returns to the blood. The Ru in rabbits has been studied both under normal and pathological circumstances. Investigation of the normals shows 1) Ru is a logarithmic function of the urine flow within the examined range. 2) Within a given urine flow Ru is independent of the filtration rate and consequently of the concentration index. 3) No variation in the Ru could be demonstrated during high or low dosage of sodium sulphate. The findings of Effersøe (1951) were thus confirmed.

Investigation under pathological circumstances embraces the effect of changes in the morphology of the kidney produced by uninephrectomy, partial corticectomy or injections of a proteose solution, and of functional changes caused by hemorrhagic shock, dehydration or the infusion of noradrenaline.

In compensatory hypertrophy, following uninephrectomy, a slight but significant variation from the normal curve is found at the higher urine flows.

In corticectomised kidneys the changes are more distinct but still not very pronounced, considering that most of the determinations of Ru fall within the normal range of variation. In the proteose treated animals the Ru is significantly higher than the normal, but all the determinations lie within the normal range of variation.

In dehydration and hemorrhagic shock no significant variation from the normal was demonstrated.

The infusion of noradrnaline in a greater dosage than 0.025 mg/min/kg resulted in a considerable variation from the normal m of the results being far below the norm range of variation.

REFERENCES

Addis, T., Myers, B. A. & Oliver, J.: The eff of unilateral nephrectomy on the function a structure of the remaining kidney. Arch. I Med. *34*, 243, 1924.

Andersen, M. & Nielsen, K. C.: Studies on renal function under experimental hypother in rabbits. Acta med. Scandinav. *151*, 191, 19

Barcroft, J. & Brodie, T. G.: Gaseous metaboli of kidney. J. Physiol. *32*, 18, 1905.

Bergstrand, A.: Studies on the Oxford Shu Thesis. Stockholm 1952.

Bing, J.: Effect of partial corticectomy and part medullectomy on the kidneys of various ma mals. Acta med. Scandinav. Suppl. *234*, 26, 1

— Onchometric determination of the degree partial renal corticectomy. Acta Path. *36*, 1955.

— Histological changes after intraperitoneal jection of proteins and proteinhydrolysat Acta path. et microbiol. Scandinav. *23*, 1946.

— Bjering, T.: Individual variations in the re tion between urea clearance and creatinin cl rance. Acta med. Scandinav. *93*, 318, 1937.

— Sperber, I. & Teilum, G.: Nephrosis-l renal changes and other effects of intrap toneal injection of protein hydrolysate. A path. *29*, 57, 1951.

Bjering, T.: Renal excretion of urea. Acta m Scandinav. Suppl. *234*, 26, 1949.

Bojesen, E.: A method for determination of in in plasma and urine. Acta med. Scandi Suppl. *266*, 275, 1952.

Brun, C.: A rapid method for determinatio paraamino-hippuric acid in kidney funct tests. J. Lab. & Clin. Med. *37*, 955, 1951.

Conway, E. J.: Microdiffusion Analysis and V metric Error. London 1947.

Corcoran, A. C. & Page, I. H.: Application diphenylamine in determination of levulos biological media; determination of inulin;

termination of levulose in small amounts of blood. J. Biol. Chem. *127,* 601, 1939.

nney, A. R.: The Secretion of Urine. London 1926.

rsøe. P.: Relation between inulin and urea clearance in rabbits with normal urine flow and increased urine flow following the administration of water. Acta physiol. Scandinav. *22,* 148, 1951.

Ratio between inulin and urea clearance in rabbits with increased urine flow following the administration of sodium thiosulphate. Acta physiol. Scandinav. *22,* 168, 1951.

Effect of sodium benzoate on the relation between inulin, creatinine, thiosulphate and urea clearance. Acta pharmacol. et toxicol. *7,* 5, 1951.

gie, J. F.: Lesser circulation of the kidney. Lancet *453,* 809, 1947.

er, J. H., Conn, H., Markley, K. & Schmidt, C. F.: Hemodynamics of renal circulation. Am. J. Physiol. *159,* 582, 1949.

er, J.: An essay toward a dynamic morphology of the mammalian nephron. Am. J. Med. *9,* 88, 1950.

Rehberg, P. B.: Studies on kidney function. II. The excretion of urea and chlorine analysed according to a modified filtration-reabsorption thory. Boichem. J. *20,* 461, 1926.

Schmidt-Nielsen, B.: Comparative aspects of urea excretion in vertebrates. Federation Proc. *15,* 531, 1956.

— Schmidt-Nielsen, K., Houpt, T. R. & Jarnum, S. A.: Urea excretion in the camel. Am. J. Physiol. *188,* 477, 1957.

Smith, H. W.: The kidney. Oxford 1951.

Study, R. S. & R. E. Skipley: Effect of renal artery-nerve stimulation on renal blood flow measured directly and by clearance and extraction of diodrast. Am. J. Physiol. *159,* 592, 1949.

Sønder, P.: Experimentelle hypertensionsundersøgelser på kaniner. Thesis. København 1953.

Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J. & Prichard, M. M.: Studies of the renal circulation. Oxford 1948.

Jarausch, K. H. & Ullrich, K. J.: Neuere Ergebnisse und Methoden zum Mechanismus der Harnkoncentrierung. Monatsschr. f. Kinderheilk. *106,* 108, 1958.

Scandinav. J. Clin. & Lab. Investigation 264—267, 13, 1961.

SERUM LACTIC DEHYDROGENASE ACTIVITY IN UMBILICA CORD, AT THE END OF LABOR IN NORMAL WOMEN, AND IN UNCOMPLICATED PUERPERIUM

By P. FYLLING

From the Department of Gynecology & obstetrics and the clinical laboratory, Drammen hospital, Drammen, Norway.

(Received for publication September 28, 1960)

The lactic dehydrogenase activity in the serum (SLDH) has been investigated in several clinical disorders (Hill & Levi 1954, Hsieh & Blumenthal 1956, McDonald, Simpson & Nossal 1957, Wacker, Ulmer & Vallee 1956, White 1956, Wroblewski 1959). It is indicated that the high serum activity in myocardial infarction and other necrotizing processes is due to the liberation of the enzyme from necrotizing tissues.

SLDH seems to be at a fairly equal both in women and men. However, Hagerman & Wellington (1959) found minor variations during the menstrual cycle with the minimum activity about ovulation time.

In recent years there have been some investigations into the lactic dehydrogenase activity (LDH) during pregnancy and delivery, but few investigations into the activity during puerperium, and the results and conclusions are not uniform. Thus Hill & Levi (1954) found an increased activity up to delivery throughout the last 6 weeks of gestation. Contrary to these, other reports indicate that the activity is slightly elevated or within normal limits throughout pr nancy. McDonald *et al.* thus found that SLDH was unchanged in 4 or 5 cases West & Zimmerman (1958) in 68 of cases. The reports of Linton, Miller & W ston (1959) and Knudsen, Cornatzer, Mo & Nelson (1958) indicated no increase SLDH in a group of 30 and 100 cases spectively, compared to groups of healt nonpregnant individuals. Comparative st of SLDH in material and fetal serum, m by Lapan & Friedman (1959), revealed per cent higher activity in fetal than maternal serum.

Thus there has been some disagreemen the reports on SLDH ante- and post-part and little attention has been focused on enzyme levels in maternal sera during p perium and in umbilical cords. The pre work is an attempt to throw some light the following problems: 1. Is there an creased SLDH at delivery and/or during puerperium? 2. Is there any correlation tween the activity in maternal serum an serum from the umbilical cord?

MATERIAL AND METHODS

SLDH was investigated in blood samples from women, 18—30 years old, with uncomplicated iveries after normal pregnancies. The first blood nple was obtained during delivery. Post partum blood samples were obtained daily or every ond day until departure from the clinic usually lays post partum. SLDH in these blood samples s compared with the activity in sera from 20 lthy, nonpregnant women, between 18—25 years age, without any special regard to the men- ual cycle.

Maternal blood was collected in test-tubes ough Wasserman needles. Centrifugation was ried out as soon as the blood coagulated, at 3000 m. for 5 minutes. The serum was pipetted off and zen within 30 minutes after collecting the blood. tal blood was obtained right after delivery in t-tubes when cutting the umbilical cord which, necessary, was squeezed into the test-tubes, and ocessed in the same way as the maternal blood. All determinations were performed within 7 ys after collection. The method used for deter- ning the LDH activity was a modification of t described by Wroblewski & La Due (1955).

Reagents: DPNH (Sigma. 92 per cent based 4 H_2O).

Na-pyruvate (Sigma).

Both reagents were kept in their original pack- gs in a refrigerator.

Phosphate buffer, 0.4 M and pH 7.4 was made from K_2HPO_4 *p.a.* and KH_2PO_4 *p.a.* and ss distilled water. The buffer was produced in sufficient amount to cover all determinations in series and was regularly controlled with a pH- ter.

DPNH-solution was made up just before deter- ning each series, of: 2.5 mg DPNH, 7 ml phos- ate buffer and 20 ml glass distilled water. A sh solution of Na-pyruvate was also made up mediately before use of: 2.5 mg Na-pyruvate d 1 ml glass distilled water. Throughout the ocedure both these solutions were kept on ice. 0.1 ml serum was added to 3 ml DPNH-solu- n, mixed and left at room-temperature for 10 nutes. This was then placed in a cuvette and ml Na-pyruvate solution added and mixed. Serial readings were taken in a Beckman spectro- photometer at 340 mμ over 5 minutes.

SLDH is expressed as units per ml per minute. One unit (E) equals a decrease in optical density of 0.001 division per ml serum per minute at 340 mμ.

RESULTS AND COMMENTS

Ass will appear from the Table I and from Fig. 1, the activity varied considerably, but there was less variation in the controls. The average activity at delivery was 637 with

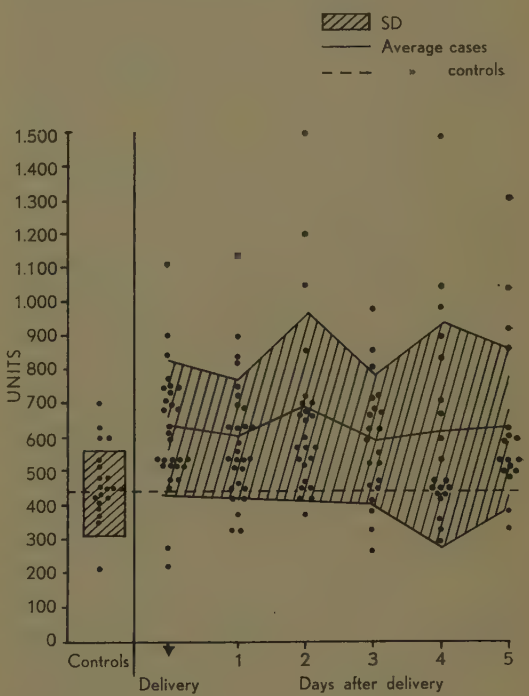


Fig. 1. Scattergram showing the distribution of LDH activity in individual cases after delivery and in controls. The shaded area shows the range of standard deviation (SD). The interrupted line shows the average activity in the controls and the dark unbroken line shows the average activity in the cases.

Table I.

	Delivery	SD	Umb. cord	SD	Controls	SD
No. of cases .	30		20		20	
Range ..	220—1100		600—1960		210—710	
Average.	637	± 193	1235	± 381	445	± 121

LDH-activity in maternal serum at delivery, in umbilical cord and in controls with standard deviation (SD).

standard deviation (SD) ± 193, and in the controls 445 with SD ± 121. SLDH at delivery was significantly higher than in the controls ($p < 0.5$). The average activity in serum from umbilical cords was 1235 with SD ± 381 and thus twice as high as in maternal serum.

The present results did not indicate any relationship between placental size and the serum activity in the umbilical cord. Neither the duration of labor nor the duration of pregnancy (in days) seemed to play any role in the activity of maternal serum or serum from umbilical cord.

The puerperal activity was fairly constant throughout the observation period; the daily levels being significantly higher than in the controls ($p < 0.5$).

Among other investigations, Persson (1959) has shown that the placenta is very rich in enzymes. It is further known that the placenta at term is a rapidly degenerating organ. As indicated in other necrotizing processes mentioned above, the liberation of placental enzymes is indicated as a possible explanation of the relatively high enzyme activity in the umbilical cord. Another possible explanation could be that fetal growth and metabolic processes increase enzyme activity in the same way as rapidly growing neoplastic tissues. There was good agre ment between the present report and t work of Hagerman & Wellington (195 which indicated that the placental barri separates the maternal from the fetal e zyme system.

It would be of interest to know how lo the activity remained at a higher level in t puerperium. The patients in this report we discharged from the clinic on the 5th or t 6th day *post partum*, and a further observ tion was not practical.

As mentioned above, no correlation b tween placental state (size, infarctions, c cium deposits *etc.*) and the enzyme activi was found. Concerning the role of the pl centa in determining the level of SLDH fetal blood, it would be value to follow the activity in fetal blood for some ti after delivery. However, for practical-clini reasons such an investigation was abandone

SUMMARY

1. The serum lactic dehydrogenase ac vity (SLDH) was determined in matern sera at delivery and in sera from umbili cords. The maternal serum was also det mined up to 5 days after delivery.

The results indicated slightly increased vity in maternal sera at delivery and after very during the whole observation peroid lays).

The average activity in sera from the bilical cord was twice as high as in mater- sera. There seemed to be no correlation veen the activity in maternal serum and erum from umbilical cord.

REFERENCES

, B. R. & Levi, C.: Elevation of a serum component in neoplastic disease. Cancer Research *14,* 513, 1954.

erman, D. D. & Wellington, F. M.: Serum lactic dehydrogenase activity during pregnancy and in the newborn. Am. J. Obst. & Gynec. *77,* 348, 1959.

eh, K. M. & Blumenthal, H. T.: Serum lactic dehydrogenase levels in various disease states. Proc. Soc. Exper. Biol. & Med. *91,* 626, 1956.

dsen, R. G., Cornatzer, W. E., Moore, J. H. & Nelson, W. W.: Serum lactic dehydrogenase and glutamic oxalacetic transaminase activities in normal pregnancy. J. Lab. & Clin. Med. *51,* 775, 1958.

Lapan, B. & Friedman, M.: A comparative study of foetal and maternal serum enzyme levels. J. Lab. & Clin. Med. *54,* 417, 1959.

Linton, E. B., Miller, E. C. jr. & Winston, N. C.: Serum lactic dehydrogenase in pregnancy. Am. J. Obst. & Gynec. *78,* 11, 1959.

McDonald, R. P., Simpson, J. R. & Nossal, E.: Serum lactic dehydrogenase. A diagnostic aid in myocardial infarction. J. A. M. A. *165,* 35, 1957.

Perssons, B. H.: Serumtransaminaser under ukompliserad graviditet. Svenska Läkartidn. *14,* 973, 1959.

Wacker, W. E. C., Ulmer, D. D. & Vallee, B. L.: Metalloenzymes and myocardial infarction: II. Malic and lactic dehydrogenase activities and zinc concentrations in serum. New England J. Med. *255,* 449, 1956.

West, M. & Zimmerman, H. J.: Lactic dehydrogenase and glutamic oxalacetic transaminase in normal pregnant women and newborn children. Am. J. M. Sc. *235,* 444, 1958.

White, L. P.: Serum enzymes. I. Serum lactic dehydrogenase in myocardial infarction. New England J. Med. *255,* 984, 1956.

Wroblewski, F.: Increasing clinical significans of alternations in enzymes of body fluids. Ann. Int. Med. *50,* 62, 1959.

— & La Due, J. S.: Lactic dehydrogenase activity in blood. Proc. Exper. Biol. & Med. *90,* 210, 1955.

Scandinav. J. Clin. & Lab. Investigation 268—275, 13, 1961.

EFFECT OF HIGH URETERAL PRESSURE ON URINE CONCENTRATION DURING WATER DIURESIS[1]

BY F. KIIL AND K. AUKLAND

From the Institute for Experimental Medical Research, University of Oslo, and Medical Department VII, Ullevaal Hospital, Oslo, Norway.

(Received for publication August 30, 1960)

It now seems well established that increase of intrapelvic pressure to 15—20 mm Hg does not influence urine flow and composition (Pilcher, Bollman & Mann, 1937; Share, 1952; Selkurt, Brandfonbrener & Geller, 1952). At higher ureteral pressures, however, the output of urine and salts decreases. From earlier studies, summarized by Cushny (1926), it is apparent that the excretion rates of urea, sulphate and phosphate were less reduced than water excretion. This suggests that the osmolar concentration of urine would increase at high ureteral pressures.

In previous stop-flow studies performed during water diuresis, water abstraction continued in the collecting ducts during the clamping period (Kiil & Aukland 1960 a, b). The osmolar concentration of urine delayed in the collecting ducts increased and might exceed plasma levels. Segments of the collecting ducts are therefore permeable to water even in the absence of antidiuretic hormone. The distal part of the collecting ducts was also found to be urea-permea

The hypothesis was advanced that medullary concentration mechanism locali in the loop of Henle operated independe of the presence of antidiuretic hormone. osmolar concentration of papillary intere tial water would decrease during water resis on account of large water abstrac from the collecting ducts. The osmolar centration due to urea would be low bec the urea concentration is low in the f delivered to the collecting ducts during w diuresis.

The water permeability of the distal t les is low during water diuresis, and the tinued sodium reabsorption from these ments during stopped flow would there render these urine portions more dilute normally. Stop-flow conditions evide exaggerate both the diluting effect in distal tubules and the concentrating effe the collecting ducts.

The present investigation was there undertaken to see if urine was dilute concentrated when continuous flow thr the nephrons was allowed. In most ex ments there was a considerable increas

[1] This study was supported by grants from Shipowner Anders Jahre, and the Norwegian Research Council for Science and the Humanities.

olar concentration of urine. This indicat- that the water permeability of segments he collecting ducts was high during these erimental conditions. The high water neability might, however, be due to ention of the collecting ducts. This pos- lity was investigated by comparing the olar concentration of urine in post- truction periods with urine from the con- kidney. The effect of ureteral pressure en the sodium concentration of urine was n during control periods was also invest- ed. On the basis of these findings the amics of water diuresis is discussed.

METHODS

Experiments were performed on ten mongrel s, weighing 12—22 kilos, anesthetised with nbutal. 1.5 meter long polyethylene tubes were rted into the ureters through a flank incision tied in place at the pelvioureteral junction so leakage along the catheters could be avoided. Water diuresis was induced by giving 5—600 water by stomach tube and was maintained ughout the experiment by the infusion of 0.45 cent sodium chloride solution at a rate of 4—8 per minute. Creatinine and p-aminohippurate AH), in amounts expected to insure maximal raction, were added to the infusion solution. ralluride (Mercuhydrin Astra) was given con- usly *i.v.* when high rates of sodium excretion e desired.

Renal pelvic pressure was increased by elevating peripheral end of the polyethylene catheter. ne from the contralateral kidney, sampled at at- pheric pressure in the renal pelvis, served as con- . Since increased pelvic pressure might have a dual effect on kidney function, urine from the erimental kidney sampled at control pressure served as control. In all experiments included his report the concentration of urine was less n 150 mMol per liter during free flow from the erimental kidney and from the control kidney ughout the experiment.

At high ureteral pressures when the urine flow was low, more than one hour was needed for equilibration. When equilibrium was assumed to be reached and urine samples obtained, the catheter was in some experiments cut 30—40 cm from the renal pelvis. The outpouring urine was sampled in 20—30 approximately equal portions. New filtrate was signified by the appearance of inulin administered *i.v.* 45 seconds before the outpouring period.

Blood and urine samples were analyzed for urea, creatinine, PAH, sodium, potassium and chloride by methods previously described (Kiil & Aukland, 1960 a, b). In lack of an osmometer, the osmolar concentration of blood and urine was estimated as the osmolar concentration of urea and creatinine, plus twice the sodium and potassium concentration measured in mEq per liter. Later comparisons of osmolar concentration of urine with osmolar values directly obtained by means of a Fiske osmometer revealed differences too small to invalidate any of the conclusions drawn in this paper.

RESULTS

When continued urine flow was allowed through the elevated catheter, the maximal intrapelvic pressure attained was regularly higher than 110 cm water, measured as the vertical distance between the tip of the catheter and the level of the renal pelvis. Since urine under these experimental conditions was usually higihly concentrated and of high specific gravity, the intrapelvic pressure was underestimated by a few centimeters. The values given are the uncorrected ones. The highest pressure attained was 134 cm water. In two experiments the addition of mannitol to the infusion fluid increased the rate of urine flow even at the maximal pressure attained, but premannitol urine flow was established by further elevating the catheter a few centimeters.

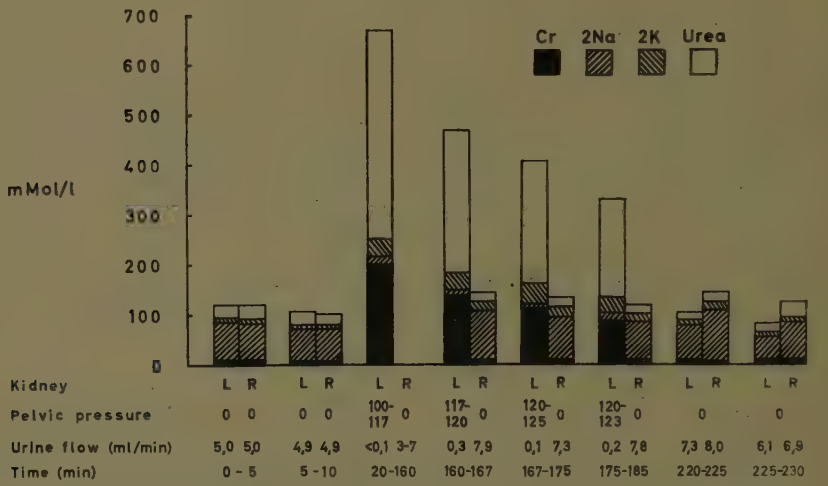
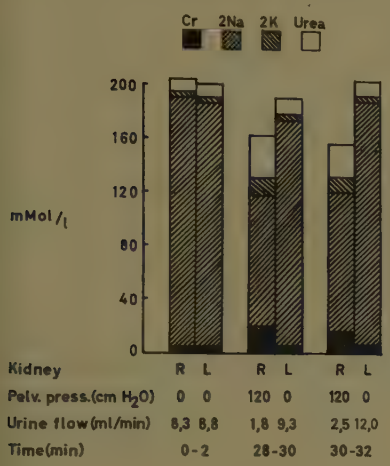


Fig. 1. Effect of increased pelvic pressure (Left kidney, L) on urine flow, osmolarity and composition during water diuresis. Right kidney (R) used as control.

At the highest ureteral pressures, change in pressure by a few centimeters was accompanied by large changes in urine flow. Variations in flow also occurred spontaneously even when the rate of urine flow from the control kidney did not change. Transient slight reduction in systemic blood pressure following additional administration of Nembrutal was accompanied by similar changes in highest obtainable ureteral pressure. Clearance determinations at high ureteral pressures were therefore unreliable, even when urine flow was kept nearly constant by small changes in pressure.

When urine flow was reduced from 5—8 ml per minute to less than 0.2 ml per minute by increasing ureteral pressure, the osmolar concentration of urine usually increased 3—5 times and exceeded the osmolar concentration of serum. On account of dead space errors even when considerable was allowed for equilibration, the maxi osmolar concentration may be estimated low. In order to avoid space errors of o site direction when sampling was desire a lower ureteral pressure, the accumul urine was emptied before the start of new equilibration period. The result typical experiment at high ureteral pres is shown in Fig. 1. At a pelvic pressur 100—117 cm water and urine flow of than 0.1 ml per minute, the osmolar con tration was seven times higher than in trols and more than twice as high as osmolar concentration of serum.

When the ureteral pressure was kept stant, increase in urine flow led to reduc in osmolar concentration. Comparison o sults from different experiments also ported the conclusion that the osmolar



2. Effect of increased pelvic pressure (Right ey, R) on urine flow, osmolarity and compo- n during merallurid diuresis superimposed on er diuresis. Left kidney (L) used as control.

tration of urine was more dependent on rate of urine flow than on the absolute ue of the ureteral pressure. The ureteral ssure required to reduce the urine flow ess than 0.5 ml per minute varied from to 125 cm water.

n most of these experiments urea was the n osmolar constituent both during free obstructed flow. The percentile in- ase in concentration was higher for atinine, so that the urea/creatinine ratio reduced when urine flow was reduced. atinine and PAH clearances were not uced at ureteral pressures up to 50 cm er, in accordance with previous findings lkurt *et al.* 1952). In some experiments re were no measurable reductions even igher pressures, but at the maximal pres- es the clearances were reduced to a few cent of control values. The concentration of potassium invariably increased, although its clearance was reduced more than that of creatinine. The concentration of sodium remained essentially unchanged at moderate reductions of urine flow, but decreased at higher pressures so that the urine was nearly sodium-free at the lowest flow rates. Usually the reduction in osmolar concentration due to sodium was more than compensated by the increase in osmolar concentration due to the other urine constituents.

This suggested that if the osmolar concentration during free flow were mainly due to sodium, the total osmolar concentration might decrease when ureteral pressure was raised. Sodium output increased when meralluride was administered, but the osmolar concentration of urine increased considerably. Fig. 2 shows the results of an experiment where the concentration, mainly due to sodium and its anions, was approximately 200 mMol per liter during free flow. At a pressure of 120 cm water the concentration was reduced to 160 mMol per liter, so that the reduction in sodium concentration outranged the increase in concentration of other constituents. A necessary pre-requisite for this occurrence seems to be that the sodium concentration is high in control urine.

Reductions in urine volume and sodium concentration precede measurable reductions in filtration rate when ureteral pressure is raised. Fig. 3 shows the result of an experiment where no reduction in creatinine clearance occurred at a ureteral pressure of 55 cm (mean value at increased pressure 52.5 ml per minute against 53.5 and 51 ml per minute in control periods before and after pressure increase). Changes in urine composition, mainly due to water abstraction,

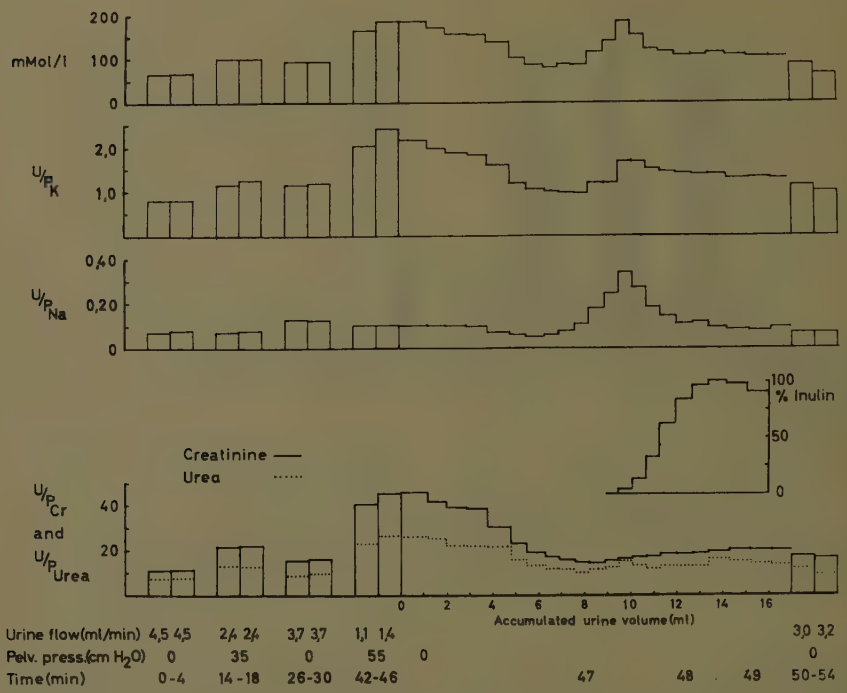


Fig. 3. Effect of moderately increased pelvic pressure on urine flow, osmolarity and composition during water diuresis. At 46 minutes sudden reduction of pressure from 55 to 0 cm H_2O followed by continuous sampling of small urine portions, in the diagram plotted against accumulated volume. Inulin injected *i.v.* 45 seconds before reduction of pressure. Urine inulin concentration recorded as per cent of maximal value.

were present already at a pelvic pressure of 35 cm water.

It was of interest to examine the outflow pattern when the ureteral pressure was suddenly reduced to control level. By plotting the concentration indices of creatinine, urea, sodium, potassium and osmolar concentration against accumulated urine volume, consistent excretion patterns were obtained. An example is shown in Fig. 3. In spite of a urine flow exceeding one ml per minute before reduction of pressure, the main characteristics of the excretion patterns were sim to those obtained in stopflow experim during water diuresis (Kiil & Aukl 1960 b). Contral levels for cretinine and concentration were reached within minute and for sodium and potassium wi four minutes. When the dead space of renal pelvis and the ureteral chateter is t into account, the creatinine concentra dropped even from the first ml obtained f the kidney, and reached control levels a same time as the dip in sodium concentra

accordance with previous stop-flow lies, the osmolar concentration pattern cribes a bimodal curve.

n experiments where the ureteral pres- e was not raised higher than 60—70 cm er, no consistent changes in the composi- and volume could be detected in the owing control periods. When ureteral ssure had been raised to the highest ainable values for one hour or more, urine n the experimental kidney was slightly otonic in the following control periods pared with that from the control kidney g. 1). Creatinine and PAH clearances e consistently reduced by 10 to 20 per t. No consistent changes in urine flow, nary composition and osmolar concentra- occurred on the control side during these eriments.

DISCUSSION

t has previously been shown that increase smolar concentration of urine in the ab- ce of antidiuretic hormone can be achieved partial occlusion of aorta or reduction of emic blood pressure (del Greco & de rdener 1956), or by compression of the al artery (Berliner & Davidson 1957). latter authors assumed that the reab- otion of sodium and water became essen- y complete farther and farther up the ule when the glomerular filtration rate reduced. By reduced delivery of pre- ne to the diluting segment less water uld be freed of solute, and only a minimal ume of solute free water would be deliver- o the final segment of the nephron res- sible for the production of hypertonicity.

A similar mechanism may contribute to the production of hypertonic urine when the ureteral pressure is raised. At the highest ureteral pressures the glomerular filtration rate was always considerably reduced. Changes in composition of urine appeared, however, at a ureteral pressure well below that where reduction in glomerular filtration rate could be detected. An attempt to explain the paradox that glomerular filtration rate may not be reduced when proximal tubular pressure is increased, is presented in the following paper (Kiil & Aukland, 1961). Selkurt *et al.* (1952) observed reduction in sodium excretion at ureteral pressures where glomerular filtration rate was not significantly reduced. They assumed that the low sodium excretion was due to indetectable reduction of glomerular filtration rate. Share (1952), making similar observations, suggested that a contributory factor might be a more sluggish flow through the tubules favoring an increase in sodium and water reabsorption.

The importance of contact-time is well visualised in stop-flow experiments. The reduction in sodium concentration in distal tubules indicates that the maximal concentration gradient for sodium is not reached in this segment during free flow even when water reabsorption is reduced on account of the osmotic effect of mannitol. A similar mechanism will be operating at increased ureteral pressure in spite of continuous flow. Reduction of pressure to control levels is followed by an outpouring of urine due to distention of the tubules (Kiil & Aukland, 1960 a). In the presence of increased tubular volume the passage time will be longer than normal.

When, during water diuresis, the ureteral pressure is high, the increase in contact time with the distal tubules will lead to a more complete sodium reabsorption. Since the distal tubules in the absence of antidiuretic hormone are relatively impermeable to water, the reduction in volume will not be appreciable. A more dilute urine is accordingly produced when the flow through the distal tubules is slow. The dilution effect will be greater at high ureteral pressure when sodium concentration in control urine is high, as it is when meralluride has been administered. The dilution effect may then be greater than the concentrating effect, and the osmolar concentration of urine will decrease as shown in Fig. 2. This illustrates the importance of water permeability of the distal tubules for the concentration of urine. High ureteral pressure will therefore have a less concentrating effect during water diuresis than during salt diuresis, when the fluid of the distal tubules is in osmotic equilibrium with surrounding interstitial fluid whether the flow rate is high or low.

Increase in ureteral pressure may reduce the rate of blood flow through the renal medulla, so that the medullary concentration mechanism may operate more efficiently. Medullary blood flow, however, would be reduced whether urine was diluted or concentrated, and is therefore not a decisive factor. Since this factor can be ruled out, the final osmolar concentration of urine probably depends upon the flow rate and hypotonicity of the fluid delivered to the collecting ducts. It remains unknown how much water has to be abstracted from the collecting ducts in order to render the interstitial fluid of the papilla sufficiently dilute. If fluid of osmolar concentration of 50 mOsm liter is delivered to the water-permeable s ment, abstraction of half the volume does increase osmolar concentration to more t 100 mOsm per liter. The osmolar concent tion of interstitial fluid of the papilla is p bably higher than that of plasma even dur water diuresis (Ullrich, 1959). Equilibri is therefore not reached. This might be to a limited water permeability in all s ments of the collecting ducts in the abse of antidiuretic hormone. It seems more sonable, however, that osmotic equilibri is not reached on account of the high fl rate through the collecting ducts dur water diuresis.

The highest osmolar concentration evidently reached in the inner zone medulla when flow rate is reduced dur water diuresis. The osmolar concentrat of interstitial water seems to fall in the di tion of the papillary tip (Ullrich, 1959). T supports the hypothesis that the larg water abstraction during water diuresis ta place in the papilla. The problem which mains to be settled is whether this h water permeability is an artefact produ by distention of the collecting ducts whether high water permeability is pres during free water diuresis. Short periods increased ureteral pressure do not lead measurable changes of renal function. If collecting ducts became more permeable water on account of distention, the osm concentration of urine in postexperime periods would be higher than that from control kidney. Reduction in glomerular ration rate would have a concentrating eff The finding of an even more dilute u suggests that protracted distention ren

distal tubules and collecting ducts less
neable to water. It is therefore concluded
the distal parts of the collecting ducts
under normal conditions are highly per-
ble to water even in the absence of anti-
etic hormone.

SUMMARY

When urine flow was reduced to less than
ml per minute by raising ureteral pres-
in dogs during water diuresis, the os-
ar concentration increased to 5—700
ol per liter. The osmolar concentration
rea, creatinine and postassium increased,
reas sodium concentration remained un-
nged at low ureteral pressures and de-
sed at high pressure. When sodium and
nions constituted the main part of urine
olarity, as after administration of meral-
le, total osmolar concentration might
ease at high ureteral pressures. Studies
he outflow pattern following sudden re-
ion of pressure to control levels showed
the reduction in sodium concentration
due to a more complete sodium rearb-
otion in the distal tubules, which remain-
essentially water-impermeable at high
eral pressures. The main additional
er abstraction during increased ureteral
ssure was shown to take place in the
ecting ducts. Increased osmolar concen-
ion of urine was observed even at ure-
l filtration rate was not reduced. Pro-
ted distention of the nephrons reduced
glomerular filtration rate in subsequent
ods at control pressure, but the osmolar
centration of urine became lower than
of the control kidney. The mechanism
vater diuresis is discussed.

ACKNOWLEDGMENTS

We thank Miss Nygaard and Miss Else-Marie Flikeid for expert technical assistance.

REFERENCES

Berliner, R. W. & Davidson, D. G.: Production of hypertonic urine in the absence of pituitary and diuretic hormone.
J. Clin. Investigation *36* 1416, 1957.

Cushny, A. R.: The secretion of urine.
Longmans, Green & Co., London, 2nd edition, 1926.

Greco F. del, & Wardener, H. E.: The effect on urine osmolarity of a transient reduction in glomerular filtration rate and solute output during a water diuresis.
J. Physiol. *131* 3507, 1956.

Kiil, F. & Aukland, K.: The role of urea in the renal concentration mechanism.
Scandinav. J. Clin. & Lab. Investigation *12* 290, 1960, a.

— Renal tubular localisation of water and sodium reabsorption in antidiuresis and water diuresis.
Scandinav. J. Clin. & Lab. Investigation *12* 277, 1960, b.

— Renal concentration mechanism and haemodynamics at increased ureteral pressure during osmotic and saline diuresis.
Scandinav. J. Clin. & Lab. Investigation *13* 1961.

Pilcher, F. Jr., Bollman, J. L. & Mann, F. C.: The effect of increased intra-ureteral pressure on renal function.
J. Urol. *38* 202, 1937.

Selkurt, E. E., Brandfonbrener, M. & Geller, H. M.: Effects of ureteral pressure increase on renal haemodynamics and the handling of electrolytes and water.
Am. J. Physiol. *170* 61, 1952.

Share, L.: Effect of increased ureteral pressure on renal function.
Am. J. Physiol. *168* 97, 1952.

Ullrich, K. J.: Das Nierenmark. Struktur, Stoffwechsel und Funktion.
Ergebn. d. Physiologie *50* 433, 1959.

Scandinav. J. Clin. & Lab. Investigation 276—287, 13, 1961.

RENAL CONCENTRATION MECHANISM AND HEMODYNAMICS AT INCREASED URETERAL PRESSU DURING OSMOTIC AND SALINE DIURESIS [1]

By F. KIIL and K. AUKLAND

From the Institute for Experimental Medical Research, University of Oslo, and Medical Department VII, Ullevaal Hospital, Oslo, Norway.

(Received for publication August 30, 1960)

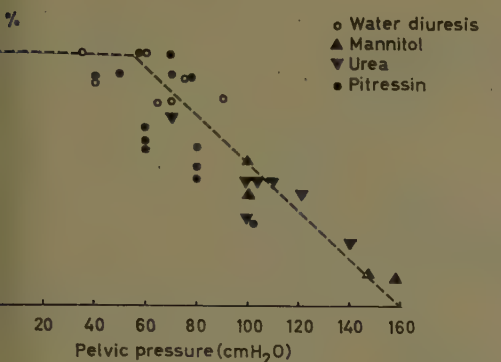
In stop-flow experiments performed during saline diuresis the osmolar concentration increased in samples delayed in the collecting ducts (Kiil & Aukland, 1960 a). The main additional water abstraction and urea accumulation, however, occurred in the distal tubules where the sodium concentration reached its minimum. Urea has been believed to add to the osmotic concentration of urine without being balanced by an osmotically equivalent amount of sodium salts in the papillary intestitium (Levinsky, Davidson & Berliner, 1959). In support of this hypothesis the distal part of the collecting ducts was found to be highly permeable to urea (Kiil & Aukland, 1960 b).

If urine was allowed to flow at reduced rate through the distal tubules and collecting ducts, the urea accumulating effect of the distal tubules and the water abstracting effect of the collecting ducts might be additive and therefore produce a marked increase in osmolar concentration. However, Levitt, Lèvy & Polimeros (1959) found that the urine osmolality fell when the filtration rate moderately reduced in dehydrated hu subjects. In experiments on dogs Levi *et al.* (1959) also showed that the osm concentration of urine fell when the glo ular filtration rate was reduced by more 30 per cent.

Malvin, Wilde & Sullivan (1958) f that renal blood flow as judged from P clearance continued uninfluenced du stop-flow periods. Kessler (1959) obse in experiments on dogs during saline diu that urine concentration decreased when flow of urine was stopped. In the fac reduced sodium load to the medulla, the osmotic concentration of the interstitial w was believed to be reduced on account washout effect by the persistent high me lary blood flow.

Contrary to this finding it is shown i present paper that osmolar concentratio creases when ureteral pressure is raised a discussion of the concentration mecha of urine an alternative to the multi countercurrent hypothesis is presented. analysis is undertaken of the relatio between renal pelvic pressure and the

[1] This study was supported by grants from Shipowner Anders Jahre, and the Norwegian Research Council for Science and the Humanities.



1. Relationship between intrapelvic pressure glomerular filtration rate in per cent of con- l values before elevating ureteral catheter.

omerular filtration and renal blood flow. ypothesis is proposed to explain why nerular filtration rate and renal blood remain rearly unchanged even at a renal ic pressure of 50—60 cm water.

METHODS

xperiments were performed on 14 anesthetised grel dogs weighing 15 to 23 kilos. Ureteral ters were tied into the renal pelvis to avoid influence of ureteral peristalsis when urine ed through the elevated catheter. In most riments the other kidney served as control, ontrol periods at normal renal pelvic pressure he experimental side were always included. ction of 20—30 sequential samples following en reduction of ureteral pressure to control was performed as previously described (Kiil ukland, 1961).

line diuresis was induced by intravenous injec- of Pitressin (Parke, Davis & Co.) at a rate 0 mU per minute dissolved in 0.45 per cent solution. Osmotic diuresis was induced by venous injection of glucose, urea or mannitol. ng high rates of urine flow hypotonic saline given in intravenous drip in order to prevent water and sodium loss. Glucose in serum rine was determined by the method of Nelson) and Somogyi (1952), mannitol by the method of Corcoran & Page (1947). Creatine, PAH, urea, sodium, potassium and chloride were determined by the methods previously listed (Kiil & Aukland, 1960, b).

Blood pressure in aorta at the level of the renal arteries and pressure in the obstructed renal pelvis were recorded by means of Statham strain-gauge manometers connected to Sanborn amplifiers. Renal arterial blood flow before and after renal pelvic obstruction was simultaneously recorded by means of a square-wave electromagnetic flowmeter. The probe was placed directly on the renal artery.

RESULTS

Fig. 1. shows the percentile reduction in glomerular filtration rate when ureteral pressure was increased to various levels during different types of diuresis. No consistent reduction in glomerular filtartion rate was observed at ureteral pressures lower than 50 cm water, and in several experiments considerably higher intrapelvic pressures could be maintained without reduction of glomerular filtration rate. Increase in ureteral pressure reduces the rate of urine flow much more during saline diuresis than during osmotic or water diuresis. Trustworthy estimations of filtration rate at high ureteral pressures were only possible during osmotic diuresis. The relationship between ureteral pressure and reduction of filtration rate seems grossly independent of type of diuresis.

Fig. 2 shows the relationship in two mannitol loading experiments between renal blood flow and renal pelvic pressure expressed in per cent of aortic diastolic blood pressure. In these experiments the renal blood flow was reduced to 40—50 per cent of control values when renal pelvic pressure was 80 per cent of the diastolic pressure; the decrease started at a renal pelvic pressure

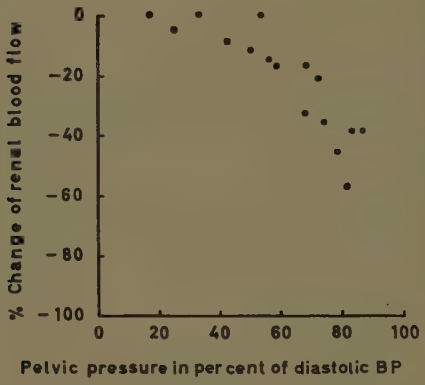
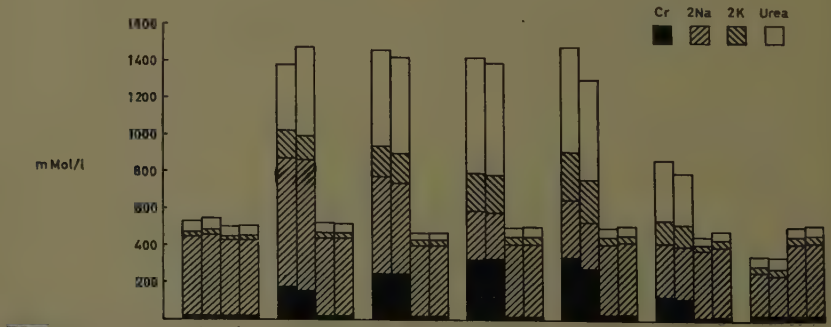


Fig. 2. Changes in renal blood flow and changes in renal pelvic pressure expressed in per cent of diastolic aortic blood pressure following complete obstruction of renal pelvis in two dogs during mannitol diuresis.

of 30—40 mm Hg. In these experiments the renal pelvis was completely occluded so that the pressure approached maximal values within 6—8 minutes. In three other experiments ureteral pressure was increased elevating the ureteral catheter and conti ous flow therefore allowed. During th experimental conditions the renal blood fl remained constant or even increased u ureteral pressures of 70 to 80 cm water. further increase of ureteral pressure, re blood flow decreased to 20—30 per cent control value. At maximal ureteral pr ures, aortic diastolic blood pressure approached but mean blood pressure never reached. At the highest ureteral pr ures there was close correlation betw changes in blood pressure and changes intrapelvic pressure. Pulse pressure transmitted without considerable damp

The highest ureteral pressures w recorded during mannitol diuresis (15 160 cm water) but pressures not more t 10—15 cm lower were recorded during w diuresis. During saline diuresis pressu higher than 100 cm water were ra recorded, and in most cases the urine f



Kidney	L	R	L	R	L	R	L	R	L	R	L	R	L	R
Pelvic pressure (cmH_2O)	0	0	60	0	60	0	100	0	80	0	50	0	0	0
Urine flow (ml/min)	3,6	4,0	0,5	4,3	0,3	5,5	0,15	4,2	0,2	3,3	0,5	4,4	2,9	2,9
C_{Cr} (ml/min)	64	62	64	68		68	35	63	47	56	53	66	49	55
C_{PAH} (ml/min)	126	124	149	138		148	103	139	126	119	127	140	122	129
Time (min)	0 - 10		30 - 40		72 - 82		130 - 140		160 - 170		185 - 195		210 - 220	

Fig. 3. Effect of increased ureteral pressure on urine composition during saline diuresis in the presence of antidiuretic hormone.

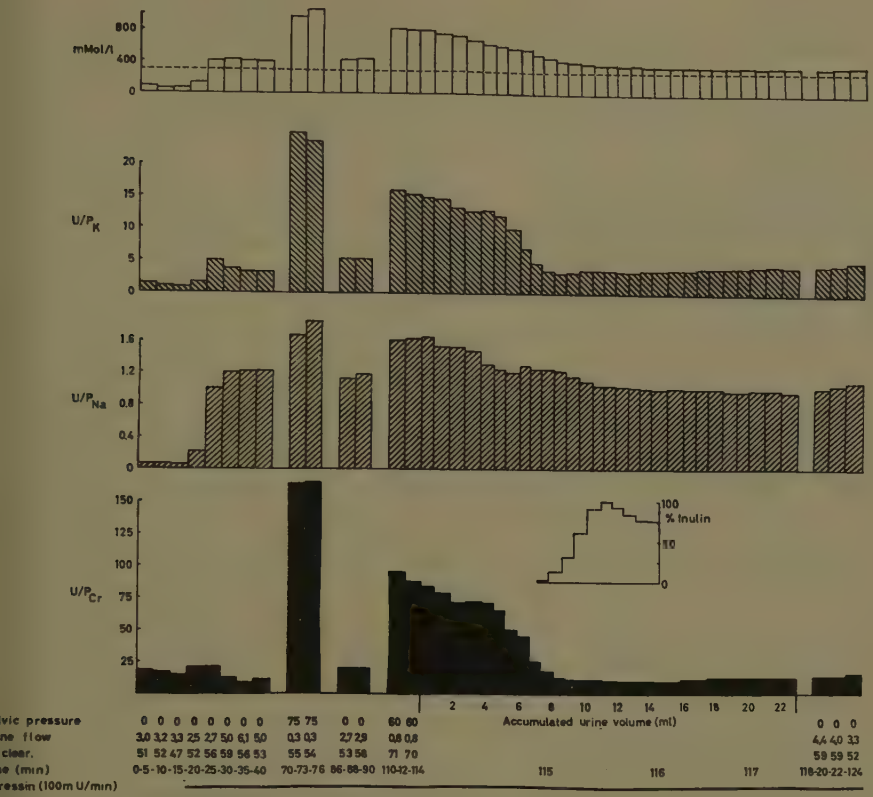


Fig. 4. Outflow patterns following reduction of ureteral pressure from 60 to 0 cm (atmospheric pressure). Inulin injected *i.v.* 40 seconds before start of sampling the "accumulated" urine volume. Preceding periods demonstrate effects of ureteral pressure and Pitressin (Parke Davis & Co) on osmolar concentration and urine flow (tabular part).

reduced to zero at ureteral pressures of -80 cm water.

When ureteral pressure was elevated dur- saline/pitressin diuresis, osmolar concen- ion of urine increased to 1400—1500 sm per liter. Reduction in urine flow n 0.3 to 0.1 ml per minute resulted in ll further increase in osmolar concen- ion of urine. The osmolar concentra- remained unchanged when urine flow was reduced from 0.5 to 0.15 ml per minute by increasing ureteral pressure (Fig. 3). The composition of urine, however, changed. The urine contained less sodium at high ureteral pressures, whereas the concentration of urea, potassium and creatinine increased so that the total osmolar concentration remained constant or increased slightly. In post-experimental control periods the osmolar concentration was lower than in urine

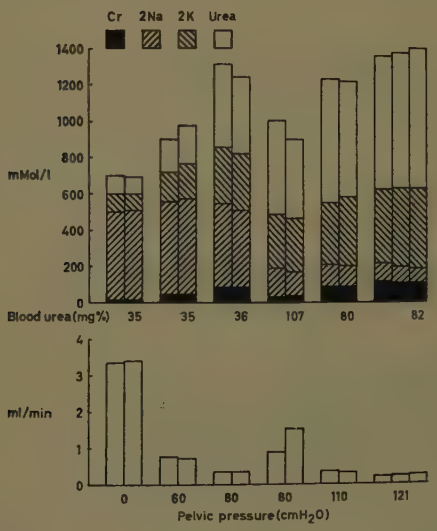


Fig. 5. Effect of increasing blood urea concentration at raised ureteral pressure.

from the control kidney. This reduction was mainly due to a decreased output of sodium and its anions and was accompanied by a reduction of the glomerular filtration rate.

When, following a short period of moderately elevated pressure, urine was allowed flow out by lowering the tip of the cathe to control level, the osmolar concentrati of urine reached control values within t minutes. Inulin as an indicator of new f rate was injected 40 seconds before pressure was reduced. It is apparent fr Fig. 4 that the composition of urine app ached control values as soon as urine due new filtrate appeared. The concentration urea remained high longer than the conc tration of the other urine constituents wh were determined (Aukland, 1961). Ur osmolarity decreased from the first samp obtained from the kidney. In the tabular p of Fig. 4, the diuretic and natriuretic eff of large doses of Pitressin (Parke Davis Co.) is demonstrated. Following a sh period of reduction in urine flow a na uresis and high urine flow was produ which could be maintained for hours.

Fig. 5 shows the effect of inducing a u diuresis when ureteral pressure was 80 water. Increase in urine flow was acc

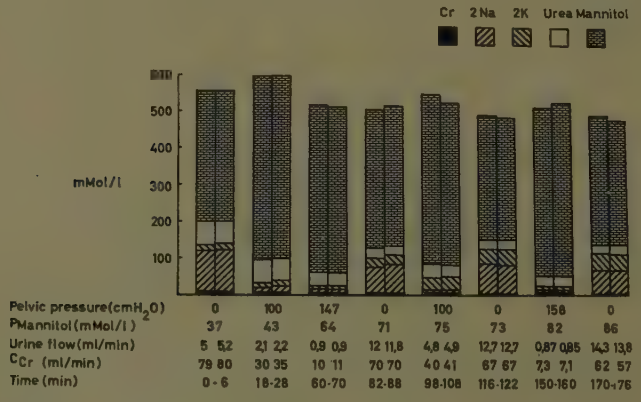


Fig. 6. Effect of increasing ureteral pressure during mannitol diuresis.

ied by a large reduction in sodium con-
ration so that the total osmolar concen-
ion of urine fell. By further increase of
eral pressure, the urine flow before urea
inistration was re-established. Osmolar
centration was now mainly due to urea
remained essentially unchanged by
her reduction of urine flow.

A total of six experiments was performed
ing large osmotic diuresis induced by glu-
e, urea or mannitol. During these experi-
ital conditions increase of ureteral press-
with ensuing reduction in urine flow had
ll or no effect on osmolar concentration
urine. Fig. 6 shows the results of an ex-
iment performed during mannitoldiuresis
h plasma mannitol concentration of 70—
mMol per liter ($P_{Mannitol}$). Ureteral
ssure of 158 cm did not increase osmolar
centration of urine above control levels
500 mMol per liter. At the highest press-
s the urine became essentially sodiumfree
the concentration of potassium was re-
ed.

t has not in any of these experiments
n possible to reduce osmolar concentra-
n by further elevation of ureteral pressure.
t on account of the considerable equili-
tion time needed, urine flows lower than
ml per minute have not been examined.
the highest ureteral pressures (Fig. 6)
glomerular filtration rate was reduced to
per cent or less of control values.

DISCUSSION

Mechanism of urine concentration.

When ureteral pressure is increased the
v rate in the tubules and collecting ducts
reduced by the mechanism outlined in the preceding paper (Kiil & Aukland, 1961). The thick ascending limb of Henles loop evidently is water-impermeable even in the presence of antidiuretic hormone. This is apparent from the micropuncture studies of Gottschalk & Mylle (1959), who found that the fluid delivered to the distal tubules was highly hypotonic during saline diuresis, whereas the hypotonicity was less pronounced during osmotic diuresis. Reduction in flow rate through the thick ascending limb therefore probably would lead to more complete sodium reabsorption and reduction in osmolar concentration of the tubular fluid. Quantitatively the effect of flow reduction would be greatest during osmotic diuresis, if it is assumed that an almost constant amount of sodium is reabsorbed from the thick ascending limb per unit of time.

In the distal tubule water will be abstracted until osmotic equilibrium with the surrounding interstitial fluid is reached. When flow rate is reduced on account of raised ureteral pressure, the reabsorption of sodium in this water-permeable segment will further reduce the volume of isotonic fluid delivered to the segment of concentration.

It now seems well established that urine is made hypertonic in the collecting ducts by equilibration with hypertonic fluid in the medulla of the kidney. How hypertonicity is created remains unknown, but the hypothesis of the multiplier countercurrent system, introduced by Hargitay & Kuhn (1951) has been widely accepted.

The present studies showed that the osmolar concentration of urine increased or remained constant when ureteral pressure was further increased, even when glomerular filtration rate was considerably reduced. The

sodium load delivered to the medulla within these limits did not seem to be the decisive factor for concentration of urine.

The multiplier countercurrent hypothesis implies that the "single effect", the sodium reabsorption only can take place against a small concentration gradient at all levels of the loop. High interstitial concentration is built up by the multiplication of this effect, during continuous flow through Henles loop. The optimal flow rate is not known, but the results of the present studies would suggest that further concentration occurs at much lower than normal flow. Furthermore, the multiplier countercurrent hypothesis implies that the ascending and descending thin limbs have different functional properties, although there is no experimental or structural evidence in favor of this. Kiil & Aukland (1960) assumed that the only water-impermeable segment of Henles loop was the thick ascending limb, and that sodium reabsorption from the collecting ducts contributed to the further increase in osmolar concentration in the interstitial fluid of the renal papilla. The maintenance of hypertonicity, even when the delivery of sodium to the collecting ducts is very low, makes it less likely that high concentration of sodium in the inner zone is due to sodium reabsorbed from the collecting ducts.

Berliner, Levinsky, Davidson & Eden (1958) presented the highly plausible hypothesis that both the ascending and descending thin limb reabsorbed sodium as the rest of the nephron, but postulated that the thin limb was almost impermeable to water. This additional assumption has been invalidated by the findings of Gottschalk & Mylle (1959) who found that the fluid in the thin limb at the turning point of the loop w also hypertonic.

In a previous communication (Kiil, 196 an alternative to the multiplier counterc rent hypothesis was presented. It was assu ed that the thin limbs of Henles loop, like t proximal tubules, were highly permeable water independent of the action of an diuretic hormone, and that sodium could actively transported out of the descendi and ascending thin limb against an elect chemical gradient which was as small as, smaller than that of the proximal tubul The reabsorbate would accordingly be osmotic equilibrium with the fluid in t lumen and the fluid in the adjacent int stitial tissue. When free water, by mecha isms outlined in the following, was remov from the interstitial fluid and osmolar co centration accordingly increased, wa would be abstracted from the loop fluid un osmotic equilibrium was reached. Sodi reabsorption would continue as long as t sodium concentration in interstitial fluid w not higher than in the fluid in the lumen a the osmolar concentration of the reabsorb would be identical with that of the loop flu If, for instance, the osmolar concentration and outside the tubular wall was 1200 mO per liter, the osmolar concentration of reabsorbate would also be 1200. Isomo sodium reabsorption would therefore co tribute to the maintenance of a high osmo concentration which primarily was due abstraction of free water.

The abstraction of free water from the t limbs of Henles loop may occur at two leve

1) Water is abstracted in the outer z where the descending thin limb runs para to the thick ascending almost waterimperm

e limb. The osmolar concentration of the bsorbate from this thick ascending limb st be high, since the fluid delivered to the al tubules is dilute. The maximal concen-ion gradient against which sodium can be bsorbed probably is so high that sodium is bsorbed from this segment at an almost stant rate under all types of diuresis.

2) Water is abstracted from the thin limb the inner zone where the osmolar concen-tion of the interstitial fluid is raised by a. As now seems well established (Kiil & kland 1960 a, b) the distal part of the ecting ducts is highly permeable to urea. If thin limb of Henle's loop is less permeable urea, urea will be osmotically active over s cell membrane and draw water from the p with ensuing increase in osmolar con-tration of the loop fluid. The isomotic bsorbate due to sodium transport would w a parallel increase in osmolar concen-tion.

When the interstitial sodium concentration thus increased, more water will be ab-acted from the collecting ducts. This is owed by a further increase in urea con-tration in the collecting duct fluid and in interstitial fluid of the papilla. The lium concentration of the reabsorbate m the loop of Henle is therefore further reased. This dual mechanism might be ponsible for very high osmolar concen-tions in the inner zone. Since the loop of nle according to the data of Gottschalk *al.* (1960) is partly permeable to urea, a ncentration gradient for urea is finally ablished when nearly as much urea is lost the fluid of the loop as is removed from the lecting ducts. The loops of the inner zone y therefore have a higher concentration index for urea than for creatinine. A high urea/creatinine ratio may be present in the distal tubules of these nephrons, whereas it will be lower in distal tubules of cortical nephrons and nephrons which only dip into the outer zone of medulla.

In the presence of antidiuretic hormone free water is accordingly abstracted from the nephrons at several levels: 1) from the thin limb of Henle's loops by the mechanism outlined; 2) from the distal tubules where the hypotonic fluid delivered from the thick ascending limb become isotonic; 3) from the collecting ducts where free water abstraction leads to hypertonicity (Fig. 7). The free water abstraction will be much larger from all these levels than during water diuresis, except from the thin limb in the outer zone and from the collecting ducts in the inner zone of medulla (Kiil & Aukland, 1961). Antidiuretic hormone seems to influence water permeability only in the distal tubules and collecting ducts in the outer zone. When ureteral pressure is increased, free water abstraction from the distal tubules will be increased because reduced flow rate through the thick ascending limb has led to a more complete reabsorption of sodium and its anions.

This also happens during mannitol diuresis. During osmotic diuresis, however, it was shown that osmolar concentration did not increase further when ureteral pressure was raised, in spite of reduction of load delivered to the site of concentration. As discussed in the preceding paper (Kiil & Aukland, 1961) the maximal electrochemical gradient for sodium reabsorption in the proximal tubules is probably reached during free flow. The maximal gradient in the thin

limbs of Henle may be even lower, and no sodium will be reabsorbed from the thin limbs during osmotic diuresis. Sodium reabsorption may, however, continue at unchanged speed from the thick ascending limb because the maximal concentration gradient is much higher in this segment. Most of the water abstraction from the isotonic urine delivered to the collecting ducts probably occurs in the outer zone and is due to the interstitial hypertonicity created by sodium reabsorption from the thick ascending limb. When the flow rate is high through the collecting ducts, as it is during osmotic diuresis, the increase in osmolar concentration will be less. More water will therefore be abstracted per unit of time (Kiil 1960). Since no sodium is reabsorbed from the thin limbs of the inner zone, the osmolar concentration will not be higher than in the outer zone, in accordance with results of tissue analyses (Malvin & Wilde 1959). Reduction of the flow rate through the collecting ducts in the inner zone will therefore not lead to increase in osmolar concentration of the urine.

Evidently, medullary concentration of urine can be achieved without the operation of a multplier countercurrent system, whereas there seems no reason to assume that solutes are trapped and water disposed of by other means than passive exchange countercurrent of medullary capillaries.

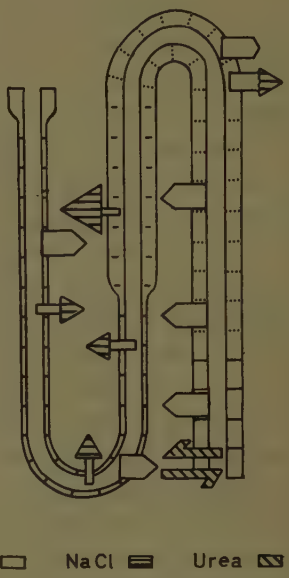


Fig. 7. Schematic drawing of Henle's loop and distal part of nephron indicating sites of free water abstraction (white arrows) during antidiuresis. Distal tubulus and collecting duct in outer zone are water permeable only in presence of antidiuretic hormone (dotted "cell" intervals). Thin limb of Henle's loop and collecting duct in inner zone are waterpermeable independent of antidiuretic hormone (unbroken intervals). Thick ascending limb is relatively water impermeable (broken intervals).

Renal haemodynamics

Numerous attempts have been made by previous authors to relate the maximal pressure in the obstructed renal pelvis to the glomerular filtration pressure, and values ranging from 40 to 110 mm Hg have been found. Assuming a pressure difference over the glomerular capillary wall of 25 mm Hg a maximal intretubular pressure of 45 mm Hg would fit with an estimated pressure in the glomerular capillaries of 70 mm Hg. The maximal intrapelvic pressure measured during this study is accordingly considerably higher than any reasonable estimate of the glomerular filtration pressure. The influence of ureteral activity can be excluded simply because the catheter was tied in the pelvic ureteral junction. Studies in dog and man have shown that the activity of the smooth

uscle cells of the renal pelvis only results small, often not measurable, pressure uctuations (Kiil, 1957).

Fig. 2 shows that the rate of blood flow reduced by 50 per cent when the intraelvic pressure is 80 per cent of the diastolic ortic blood pressure. The difference in mean ressure between aorta and the glomerular apillaries is therefore:

(Diastolic pressure + ⅓ pulse pressure) - (0.8 diastolic pressure + colloid osmotic ressure). This is roughly 0.2 times the diaolic blood pressure. This is the pressure ifference when the renal blood flow is half ne normal value. The pressure drop will be vice as large at normal renal blood flow the resistance in the preglomerular vessels emains unchanged. With a mean aortic lood pressure of 100 mm and a diastolic lood pressure of 80 m Hg, the normal lomerular capillary pressure would accordgly be 68 mm Hg. Intrapelvic pressures igher than the normal glomerular pressure ay therefore be intepreted as a consequence reduction in intrarenal blood flow without aking additional assumptions.

However, Figs. 1 and 2 show that intraelvic pressure may be 50 cm water or more ithout any reduction in glomerular filtrat-n rate and renal blood flow. These obser-ations are in accordance with previous earance determinations (Selkurt, Brand-nbrener & Geller, 1952).

Gottschalk & Mylle (1956) suggested that e parallel rise in peritubular capillary pressre and tubular pressure at intrapelvic pressre up to 35—40 mm Hg was due to comression of intrarenal veins and venules. ince renal blood flow, as shown in the preent study, is not reduced at this intrapelvic pressure, the total resistance of the intrarenal vessels has remained unchanged. Since tubular and capillary pressures are almost identical, the interstital pressure is probably of the same order of magnitude. The transmural pressure, which is the distending pressure, will therefore remain unchanged. The hypothesis is therefore advanced that if interstitial fluid pressure is transmitted to the capillaries, there will be no change in transmural pressure and the resistance will remain unchanged.

Gottschalk & Mylle (1957) found proximal tubular pressures of 10—15 mm Hg in rats. Assuming a pressure in the glomerular capillaries of 70 mm Hg, it follows that the main resistance to flow of preurine through the nephron is probably localised in the glomerular membrane, where the pressure drop would be of the order of 30 mm Hg. When tubular and intracapsular pressures were increased, there would be a similar increase in pressure in the glomerular capillaries. The blood flow through the capillaries and the glomerular filtration rate would remain unchanged. In spite of the large pores of renal capillaries, one might expect that pressures were not completely transmitted, and that there would be a tendency to reduction in blood flow and filtration rate at all levels of ureteral pressure.

The main resistance in the renal vascular system is localised in the pre- and postglomerular arterioles. It is conceivable that the tone of this muscular structure is a function of the transmural pressure. Increase in blood pressure leads to constriction so that the blood flow remains essentially constant (renal auto-regulation). Increase in interstitial fluid pressure would be more poorly

transmitted through the muscular structures of the preglomerular arterioles than through the wall of the capillaries. The transmural pressure would therefore be reduced. Increase in interstitial pressure may accordingly have the same effect as reduction in blood pressure: dilatation of the arterioles. This hypothesis is advanced in order to explain that in a few of our experiments an increase in blood flow was observed up to a ureteral pressure of 80 cm water.

SUMMARY

Rate of urine flow during saline diuresis in the presence of antidiuretic hormone was reduced in 14 anesthetised dogs by increasing ureteral pressure. Urine concentrations of 1400—1500 mMol per liter were reached. Reduction in urine flow to less than 0.3 ml per minute by further increase of ureteral pressure did not lead to significantly higher urine concentration. During mannitol diuresis osmolar concentration remained almost constant at all levels of pressure. At the highest obtainable pressures (150—160 cm water) the urine was almost sodium-free.

An alternative to the multiplier countercurrent hypothesis for the concentration of urine is presented, assuming that the thin limbs of Henle's loop are water-permeable and able to transport sodium only against a small concentration gradient.

Recording by means of an electromagnetic flowmeter showed that renal blood flow remained constant (or increased slightly) up to ureteral pressures of 70—80 cm water. On further increase in pressure, renal blood flow decreased to 20—30 per cent of control values. Clearance determinations showed that ureteral pressure might be increased to more than 50 cm of water without consistent reductions of glomerular filtration rate. The constancy of blood flow and glomerular filtration rate at moderate increase of ureteral pressure is assumed to be due to unchanged transmural pressure in the water-permeable renal capillaries.

ACKNOWLEDGMENTS

We wish to thank Miss Aud Vogt for mannitol determinations and cand. real. J. Krog and civil engineer S. Leraand for their help in electromagnetic blood flow recordings.

REFERENCES

Aukland, K.: In preparation.

Berliner, R. W., Levinsky, N. G. Davidson, D. G. & Eden, M.: Dilution and concentration of the urine and the action of antidiuretic hormone. Am. J. Med. *24*, 730, 1958.

Corcoran, A. C. & Page, I. H.: Method for determination of mannitol in plasma and urine. J. Biol. Chem. *170*, 165, 1947.

Gottschalk, C. W. & Mylle, M.: A micropuncture study of pressure in the proximal tubulus and peritubular capillaries of the rat kidney and their relation to the ureteral and renal venous pressure. Am. J. Physiol. *185*, 430, 1956.

— Micropuncture study of pressures in proximal and distal tubules and peritubular capillaries of the rat kidney during osmotic diuresis. Am. J. Physiol. *189*, 323, 1957.

— Micropuncture stydy of the mammalian urinary concentration mechanism: evidence for the countercurrent hypothesis. Am. J. Physiol. *19*[illegible] 927, 1959.

— Lassiter, W. E., Mylle, M., Ullrich, K. J., Schmidt-Nielsen, B., Pehling, G. & O'Dell, R.: Micropuncture study of the composition of fluid from loops of Henle and collecting ducts in the rodent papilla. Excerpta med. 2[illegible] 43, 1960.

rgitay, B. & Kuhn, W.: Das Multiplikationsprinzip als Grundlage der Harnkonzentrierung in der Niere. Z.schr. f. Electrochemie *55*, 539, 1951.

ssler, R. H.: Reduction of chloride excretion by artificially lowering the chloride concentration in the renal papilla. Physiologist *2*, 70, 1959.

l, F.: The Function of the Ureter and Renal Pelvis. W. B. Saunders Co., Philadelphia, 1957.

- The mechanism of concentration of urine. An alternative to the countercurrent multiplier hypothesis. J. Oslo City Hosp. *10*, 245, 1960.

l, F. & Aukland, K.: Renal tubular localization of water and sodium reabsorption in antidiuresis and water diuresis. Scandinav. J. Clin. & Lab. Investigation *12*, 277, 1960 a.

- The role of urea in the renal concentration mechanism. Scandinav. J. Clin. & Lab. Investigation *12*, 290, 1960 b.

- Effect of high ureteral pressure on urine concentration during water diuresis. Scandinav. J. Clin. & Lab. Investigation *13*, 1961.

Levinsky, N. G., Davidson, D. G. & Berliner, R. W.: Effects of reduced glomerular filtration on the urine concentration in the presence of antidiuretic hormone. J. Clin. Investigation *38*, 730, 1959.

Levitt, M. F., Levy, M. S., & Polimeros, D.: The effect of a fall in filtration rate on solute and water excretion in hydropenic. man. J. Clin. Investigation *38*, 463, 1959.

Malvin, R. L., Wilde, W. S. & Sullivan, L. P.: Localization of nephorn transport by the stop-flow analysis. Am. J. Physiol. *194*, 135, 1958.

Malvin, R. L. & Wilde, W. S.: Washout of renal countercurrent Na gradient by osmotic diuresis. Am. J. Physiol. *197*, 177, 1959.

Nelson, N.: A photometric adaption of the Somogyi method. J. Biol. Chem. *153*, 375, 1944.

Selkurt, E. E., Brandfonbrener, M. & Geller, H. M.: Effects of ureteral pressure increase on renal haemodynamics and the handling of electrolytes and water. Am. J. Physiol. *170*, 61, 1952.

Somogyi, M.: Notes of a sugar method. J. Biol. Chem. *195*, 19, 1952.

Scandinav. J. Clin. & Lab. Investigation 288—290, 13, 1961.

THE EXCRETION OF VANILMANDELIC ACID IN THE URINE OF SCHIZOPHRENICS

By K. PIND and A. FAURBYE

From Sct. Hans Hospital, Dept. D., Roskilde, Denmark.

(Received for publication September 24, 1960)

Vanilmandelic acid (VMA = 3-methoxy-4-hydroxy-D-mandelic acid) is one of the most important metabolic products of adrenaline and noradrenaline. The infusion of adrenaline and noradrenaline labeled with radioactive carbon in animals and humans has demonstrated that only 2 to 4 per cent are excreted in the urine in the original state, while the remainder is excreted as metabolic products, including vanilmandelic acid.

The metabolism of adrenaline and noradrenaline is of increased interest to psychiatrists after Hoffer, Osmond & Smythies (1954) proposed the theory that the transformation of adrenaline to adrenochrome is the cause of schizophrenia.

The present report concerns an investigation of the spontaneous excretion of VMA in chronic schizophrenics as compared to normal control persons under standardized conditions.

Patients and Controls.

35 schizophrenics were investigated. They were from 20 to 69 years old (the average age was 51 years). The average duration of the psychosis was 20 years. The diagnosis was indisputable in all cases, on account of autism, hallucinations and delusions. Th patients had no pharmacotherapy during th investigation.

Moreover, 20 controls (employees) fro 22 to 50 years of age (average age 37 year were examined.

Patients and controls were all females.

PROCEDURE

The day before the investigation the experime tal person took ordinary diet, but had no coffe and fasted from 9 p. m. On the day of the inves gation the participant passed urine at 7 a. m. a drank 400 ml water. At 9 a. m. urine was pass again, and the urine sample from 7 till 9 o'clo was investigated. The participant was up and ca ried out her normal work during the investigatio

METHOD

The determination of the quantity of VMA w carried out essentially as described by *Armstron Shaw & Wall* (1956) by paper-chromatograph The first direction: isopropanol-water-ammoniu hydroxide (25 per cent) (40:9:1). Second dire tion: benzene-propionic-acid-water (20:14:1). T scanning of the spots after dying with diazotiz sulphanilic acid was done by a photometer a comparison with standard chromatograms cont ning known quantities of VMA, using synthe D,L-VMA which was kindly given us by M. Armstrong.

RESULTS

As will be seen from the table there was
difference in the excretion of VMA in
onic schizophrenics and normal controls
er 10 to 12 hours fasting, either as regards
ncentration, total amount or in proportion
the amount of total nitrogen excreted.
As we originally had another purpose with
s investigation, the excretion of VMA was
y determined in proportion to nitrogen in
ertain number of the participants.

DISCUSSION

Armstrong & Macmillan (1957) and Arm-
ong, Macmillan & Shaw (1957) demon-
ated that VMA normally occurs in urine,
l that approximately 30 per cent of in-
ed noradrenaline is excreted as VMA.
e major part probably originates from
radrenaline, but it may also derive from
renaline. Similar results were attained by
rschner, Goodall & Rosen (1958), Goodall
959) and Axelrod (1959).
Studnitz (1960), who uses high voltage
ctrophoretic separation of phenolic acids,
found the same normal values of the excre-
tion of VMA as we did.

Mann & La Brosse (1959) examined the excretion of VMA in 24 hour samples in normal persons and chronic schizophrenics who had the same diet during the investigation period, and found no difference between the two groups.

In agreement with these results Bergsman (1959) was not able to find any difference in the excretion of adrenaline and noradrenaline in the urine of normal persons and chronic schizophrenics when they were examined at rest. By the injection of insulin he was able to cause a rise in the excretion of adrenaline and noradrenaline but the rise was not significantly different from that of normal controls. Adrenaline and noradrenaline were determined by a biological method.

Nor did we during our investigation of the excretion of VMA in the urine find any difference between the normal persons and the schizophrenics, but this does not refute the theory of Hoffer, Osmond & Smythies (1954) which assumes that in schizophrenics

Table I. *Excretion in urine from schizophenics and controls of VMA from 7 to 9 a. m. under standardized conditions. The excretion of VMA is indicated per 2 hrs., per ml urine and in proportion to the excretion of total nitrogen. (These investigations had originally another purpose and therefore VMA was only determined in proportion to N in 20 of the schizophenics and in 11 of the controls.)*

	Schizophrenics				Controls			
	Number	Average	Maximum	Minimum	Number	Average	Maximum	Minimum
resis ml/2 hrs.	35	193	550	48	20	209	510	50
ng/2 hrs.	15	690	1720	276	9	730	1030	549
IA µg/2 hrs....	15	400	966	75	9	395	483	240
IA µg/ml.....	15	3.45	7.14	0.48	9	3.08	8.37	1.19
. VMA/N.....	35	529	1010	270	20	572	841	320

a small part of adrenaline is transformed to adrenochrome which poisons the organism and causes the schizophrenic symptoms. It has not been possible to verify this theory, as the presence of adrenochrome in the blood of schizophrenics has not been proved (Randrup & Munkvad (1960) and others).

In connection with the discussion of Hoffer, Osmond & Smythie's theory on the abnormal metabolism of adrenaline as the cause of schizophrenia it may be mentioned that Kemali (1960) found that an extract of aromatic substances (Decker's method) from the urine of schizophrenics was more toxic when injected into mice than an extract from the urine of normal persons. A critical review of the many studies of the toxidity of urine and serum from schizophrenics by different authors is given by McGeer & McGeer (1959).

SUMMARY

The spontaneous excretion in the urine of 3-methoxy-4-hydroxy-D-mandelic acid (VMA) was investigated in chronic schizophrenics and normal controls under standardized conditions. No difference was found between the two groups as to the total amount excreted, concentration in urine and the proportion of VMA to the amount of total nitrogen excreted.

REFERENCES

Armstrong, M. D. & McMillan, A.: Identification of a major urinary metabolite of norepinephrine Federation Proc. *16,* 146, 1957.

Armstrong, M. D., McMillan, A. & Shaw, K. F.: 3-methoxy-4-hydroxy-D-mandelic acid, urinary metabolite of norepinephrine Biochi Biophys Acta *25,* 422, 1957.

Armstrong, M. D., Shaw, K. N. F. & Wall, P. The phenolic acids of human urine. Pa chromatography of phenolic acids. J. Bi Chem. *218,* 293, 1956.

Axelrod, J.: The Metabolism of Catecholamin in Vivo and in Vitro. Pharmacol. Rev. 402, 1959.

Bergsman, A.: The urinary excretion of adre line and noradrenaline in some mental diseas Acta psychiat. Scandinav. *34,* Suppl. 133, 19

Goodall, McC.: Metabolic products of adrenali and noradrenaline in human urine. Pharma Rev. *11,* 416, 1959.

Hoffer, A., Osmond, H., & Smythies, I.: Schi phrenia: A new approach. J. Ment. Sc. *J* 29, 1954.

Kemali, D.: Biological effects of aromatic extra from urine of schizophrenics. Nature, Lond *185,* 540, 1960.

Kirshner, N., Goodall, McC. & Rosen, L.: Me bolism of d,l-adrenaline-2-C^{14} in the hum Proc. Soc. Exper. Biol. & Med. *98,* 627, 1

Mann, I. D. & La Brosse, E. H.: Urinary ex tion of phenolic acids by normal and schi phrenic male patients. A. M. A. Arch. gen Psychiat.: *1,* 547, 1959.

McGeer, E. G. & P. L. McGeer: Physiolog Effects of Schizophrenic Body Fluids. J. M Sc. *105,* 1, 1959.

Randrup, A. & I. Munkvad: On the Measurem of Adrenochrome in Blood. Am. J. Psychi *117,* 153, 1960.

Studnitz, W.: Methodische und klinische Un suchungen über die Ausscheidung der Methoxy-4-hydroxy-mandelsäure im U Scandinav. J. Clin. & Lab. Investigation Suppl. 48, 1960.

andinav. J. Clin. & Lab. Investigation 291—296, 13, 1961.

OXYGEN DISSOCIATION CURVE OF HUMAN BLOOD EQUILIBRATED WITH HIGH CONCENTRATIONS OF OXYGEN

By C. E. G. LUNDGREN[1]

From the Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark.

(Received for publication October 27, 1960)

Previous studies of the dissociation curve oxyhemoglobin of normal human blood at ygen tensions above 120 mm mercury have the most part been concerned with *in vivo* nditions. According to information given Nahas, Morgan & Wood (1952), who rked with a cuvette oximeter for deter- nation of oxygen saturation and polarogra- ic measurement of oxygen tensions of the od, an oxygen tension of 400 mm mercury required to produce complete saturation of moglobin *in vivo*. Results obtained by rgan, Wood & Helmholz (cited by Nahas *al.* 1952) from *in vitro* studies with a larograph are claimed to agree closely with ir *in vivo* results. Using a spectrophoto- tric method, Hickam & Frayzer (1952) served that *in vitro* saturation of blood at oxygen tension of 630 mm caused a gain in oxygen saturation of approximately 2 per cent as compared to the saturation obtained by oxygen tensions existing in air at sea level. Their results do not indicate, however, at which oxygen tension full saturation is first reached.

The present study is an attempt to increase the sparse information available on the oxygen dissociation curve of human blood equilibrated *in vitro* with oxygen tensions of above 100 mm mercury. Owing to its widespread use in research work, it seemed justifiable to try the gasometric technique described by Peters & Van Slyke (1932) for the determinations of the oxygen saturation of the blood. Precautions were taken to minimize the errors in hematocrite pointed out by Roughton, Darling & Root (1944) which may be introduced when the blood drains off the walls of a tonometer. Instead of using their correction method, dependent on blood and tonometer volumes, a bubble tonometer was used in which the wall

[1] Present address: Aero-Medical Laboratory, titute of Physiology, University of Lund, eden.

that could withhold plasma was kept in small proportions relative to the volume of the blood sample. In order to reduce the importance of inactive forms of hemoglobin, blood was taken from nonsmokers and the experimental procedure was planned so as to avoid irregularities in reactivation of such pigment.

METHOD

Sampling and equilibration. Blood was obtained from twenty healthy adult nonsmokers. The subjects are represented at random in the series of different oxygen tensions. All objects that came in contact with the blood had either been sterilized or thoroughly cleaned with detergent, hot and distilled water. Thirty ml of blood were drawn from the cubital vein into a syringe, the dead space of which contained a one per cent solution heparin. After mixing with two more drops of heparin the blood was divided into two equal parts in two 30 ml test tubes. A few drops of fluid silicone defoamer were then deposited on the surface of the blood. The test tubes were sealed off with rubber stoppers, pierced with a cannula and a 1 mm bore polyethylene tube extending to the bottom, and transferred to the saturation bath. This consisted of a water container in which water was kept at a constant temperature of 37 ± 0.15° C by means of a thermostat and circulated by means of a stirring device. The gas mixtures for the saturation of the blood samples were bubbled through water-filled test tubes which served as moistening devices and were transferred to the blood samples by the aforementioned polyethylene tube. The test tubes with their stoppers and connections were all submerged during the bubbling periods except for the cannula. Bubbling through the blood served the dual purpose of saturation and stirring. Overflowing gas escaped through the cannula in the rubber stopper. The equilibration time lasted for at least one hour which proved to be sufficient. This was indicated by the absence of any tendency of a rise in the results in the second of duplicate analyses of oxygen content in the blood samples. Gas mixtur were stored in pressure cylinders and delivere through reducing valves at a constant flow about 7 ml per minute. Barometric pressure throug out the investigation was 761 ± 10 mm mercu Two experimental series were made. *Series* Fifty sets of analyses of the type described belo were made. One blood sample was continuous bubbled through with a mixture of oxygen a 5.5 ± 0.1 per cent carbon dioxide. The oxyg capacity was derived from analyses on this bloo In the other tube, equilibration was made wi 5.5 ± 0.1 per cent carbon dioxide and varyi oxygen-nitrogen mixtures, giving oxygen-tensio between 100 and 650 mm of mercury. The blo from this tube was analyzed for oxygen conte When, in some cases, the same sample was us for equilibration with two gas mixtures the seco was always richer in oxygen than the precedin *Series II:* Fifty-one sets of analyses were mad All blood samples were first saturated with ox gen and 5.5 ± 0.1 per cent carbon dioxide. Aft 60 to 90 minutes, samples for analysis of oxyg capacity were taken and the blood then expos to one of the mixtures with varying oxygen perce tages. When samples for determination of the ox gen content constituted the second phase, the blo was finally rebubbled with the oxygen-carbon dio ide mixture for the second determination of oxyg capacity.

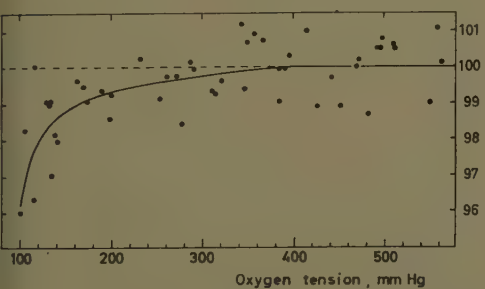
Analyses and calculations. The composition the gas mixtures was analyzed *in duplo* before ea experiment according to the technique described *Scholander* (1947) and the gas tensions calculat from barometric pressure minus the tension water vapor (47 mm mercury at 37° C).

When samples were taken for analysis of oxygen content, the test tube containing blood w lifted out of the water, and the plastic tube ext ding to its bottom was disconnected from the mo tening tube and connected to a 1 ml Ostwald pette. Blood was forced into the pipette by inject air through the cannulas over the blood surfa Care was taken to let only a few seconds ela between the interruption of the bubbling and filling of the pipette, the blood being analy

nediately afterwards. Analyses of the oxygen ıtent of the blood samples equilibrated with the ious oxygen tensions were made according to manometric method described by *Peters & Van ke* (1932). Reduction for physically dissolved ygen, expressed as volume percent, was made h the factor 0.0031 times the oxygen tension mm mercury (calculated from Table 12, page 7: *S. Dittmer & R. M. Grebe*. Handbook of Respi- ion 1958). The determinations made on blood ilibrated with the oxygen-carbon dioxide mixture re considered to give the oxygen capacity of the od. Saturation figures were obtained by expres- g the oxygen content of blood samples equili- ted at the several oxygen tensions as percentage the oxygen capacity. All figures were obtained m the averaged values of dublicate analyses of same sample with the exception of the analyses oxygen capacity in series II in which equili- tion and analysis of the oxygen content at the ver oxygen tension was interposed between two gle determinations of capacity. The analyses series I were made by a less experienced worker, ereas those of series II were handled by a highly lled technician.

RESULTS

The results from series I and II, are pre- nted in Figs. 1 and 2 respectively. Satura-



g. 1. Percentage 0_2 saturation of human whole od from 20 subjects. Blood samples directly posed to the various oxygen tensions (Cf. legend Fig. 2). Blood at about 650 mm Hg was assumed to be 100 per cent saturated.

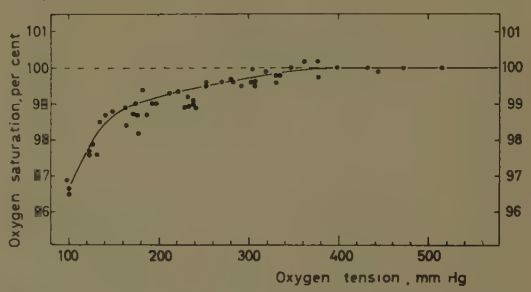


Fig. 2. Percentage O_2 saturation of human whole blood from 20 subjects. Blood samples pre-treated at about 650 mm Hg oxygen tension before exposure to the various lower oxygen tensions. Blood at about 650 mm Hg was assumed to be 100 per cent saturated.

tion percentage is plotted *versus* oxygen pressure. Each dot in Figs. 1 and 2 represents the result of duplicate analyses as described above. The curves were drawn independently. Starting with a saturation of about 96.5 per cent at an oxygen tension of about 100 mm mercury where the observation points, however, are few, the curves proceed in an asymptotical fashion to between 99 and 99.5 per cent saturation at the oxygen tension of 200 mm mercury and 100 per cent saturation at about 370 mm mercury.

DISCUSSION

The concentrating effect upon blood exerted by certain tonometers as pointed out by Roughton *et al.* (1944) seems to be unimportant in the present experiments, as the bubble tonometers used have no appreciable surface capable of withholding plasma.

The possible disturbing influence of the

presence of COHb in blood and/or gradual reversion of inactive pigments on determination of oxygen content of blood has also been stressed by Roughton *et al.* (1944). These sources of error are likely to have been avoided by using non-smokers as blood donors and by the extended equilibration times. This belief is furthermore supported by the complete agreement between the curves in Fig. 1 (from series I) and Fig. 2 (from series II). Even if the equilibration procedures at the various oxygen tensions in series I might have resulted in a varying degree of reactivation of inactive pigment, this possibility should have been eliminated in series II in which all samples were first equilibrated for more than an hour with an oxygen tension of about 650 mm mercury. The lack of difference between the two series in this respect is in full agreement with the findings of Hickam & Frayzer (1952). Any real difference in oxygen absorption of samples equilibrated at adjacent oxygen tensions due to inactive pigment would also have been diminished above 370 mm mercury in series I. The greater dispersion of the results in series I as compared to series II is mainly due to the lesser skill of the analyst of the former.

The oxygen dissociation curve within the lower range of oxygen tensions studied in the present work has been the interest of several workers (see below) and is usually regarded as well defined. Thus the saturation in arterial blood at 100 mm mercury corresponding to air breathing at sea level is reported to be 97 to 98 per cent. It is possible, however, that saturation values of too high a level might have been obtained in some instances. Bartels & Harms (1959), in experiments with *in vitro* equilibration a gasometric analyses, arrived at full satur tion at about 200 mm mercury. As realiz by them, their figures for oxygen capaci are too low, as full saturation was regard to occur at 200 mm mercury. The results Roughton and associates (1944) may ha been influenced in the same way, as th based their calculation of 97 per cent satur tion at about 100 mm mercury on determin tions of oxygen capacity made on blo equilibrated with atmospheric air. The sa circumstances may play a part in the obse vations of Wood & Cronin (1949). Th found about 98 per cent saturation at 1 mm of mercury after *in vivo* equilibrati with air. The corresponding oxygen cap cities were measured on blood saturated *vitro* with air. As pointed out by Hickam Frayzer (1952) and Bartels & Harm (1959), the oxygen capacity of blood equi brated at 150 mm mercury may gain abo 2 per cent in saturation if the oxygen te sion is increased sufficiently. An examp may show the significance of this: the ox gen content of a blood sample at a giv oxygen tension is 19.6 vol. per cent and t reference sample contains 20 vol. per cent 150 mm mercury, which yields a saturati of 98 per cent. If the oxygen capacity 20 vol. per cent is increased by 2 per cent saturation, it becomes 20.4 vol. per cent a the saturation will instead become 96.1 p cent. In determinations on whole blood fr seven subjects with a spectrophotomet method, Hickam & Frayzer found me saturations of 97.4 per cent at 146 mm H and 94.6 per cent at 97 mm Hg. The equi bration *in vitro* lasted two hours and t oxygen capacity was derived from equilibr

s at 637 mm mercury. Nahas *et al.* 52) measured oxygen content with a le blood oximeter and oxygen tension rographically on *in vivo* saturated blood. gen saturation on breathing air with an med oxygen tension in blood of 100 mm cury was found to range approximately veen 97 and 98 per cent. The blood was sidered fully saturated when its oxygen ion was 400 mm mercury or greater.

he results of the present investigation cate a rather low saturation of 96 to 97 cent at an oxygen tension of 100 mm Hg. results, however, are based on too few surements at this range of oxygen ten- to settle the question of the oxygen ration of human blood at 100 mm mer- .

arlier investigations at higher oxygen ions are for the most part in accordance the present. In their above mentioned k, Nahas *et al.* (1952), on *in vivo* satu- d blood, found about 99 per cent satura- at 200 mm mercury which is in agree- t with the present study. They state that oxygen tension of 400 mm mercury is uired to produce practically complete ration of hemoglobin *in vivo*. The same e of affairs is indicated by the *in vitro* ies of Hickam & Frayzer (1952) who d an increase of 2 per cent in saturation n blood saturated at the oxygen tension ting in air at sea level was compared to d saturated at 630 mm mercury. Among rs, Matthes and co-workers (1937), in k with an ear oximeter, have lent sup- to the results indicated above. They d an oxygen tension of 300 mm mercury ssary for complete hemoglobin satura-

SUMMARY

The present results obtained with the gasometric method of Peters & Van Slyke (1932) on whole blood equilibrated *in vitro* are in accordance with the views of various other workers. It was found that the chemically bound oxygen in human blood increases up to an oxygen tension of about 370 mm mercury where 100 per cent saturation is reached when blood equilibrated at a pO_2 of 650 mm mercury is assumed to be fully saturated. This assumption seems justified in that the average saturation is the same, 100.0 per cent, both for the 20 values in Fig. 1 and the 7 corresponding values in Fig. 2 that were obtained at tensions above 370 mm mercury.

This work was made possible by a grant to the author from Ebba og Herman Bings Legat.

REFERENCES

Bartels, H. & Harms, H.: Sauerstoffdissoziationskurven des Blutes von Säugetieren. Pflüger's Arch. f. d. ges. Physiol. *268*, 334, 1959.

Dittmer, D. S. & Grebe, R. M.: Handbook of Respiration. Philadelphia and London: Saunders, 1958.

Hickam, J. B. & Frayser, R.: Spectrophotometric oxygen determination on whole blood samples. Apparent increase in oxyhemoglobin under high oxygen tensions. J. appl. Physiol. *5*, 125, 1952.

Matthes, K., Queraltó, J. G. & Malikiosis, X.: Arch. f. exper. Path. u. Pharmakol. *185*, 622, 1937.

Morgan, E. H., Wood, E. H. & Helmholz, H. F. Jr.: Unpublished data.

Nahas, G. G., Morgan, E. H. & Wood, E. H.: Oxygen dissociation curve of arterial blood in men breathing high concentrations of oxygen. J. appl. Physiol. *5*, 169, 1952.

Peters, J. B. & Van Slyke, D. D.: Quantitative Clinical Chemistry Methods. Baltimore: Williams and Wilkins, 1932.

Roughton, F. J. W., Darling, R. C. & Root, W. S.: Factors affecting the determination of oxygen capacity, content and pressure in human arterial blood. Am. J. Physiol. *142,* 708, 1944.

Scholander, P. F. J.: Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. J. Biol. Chem. *167,* 2 1947.

Wood, E. H.: Normal oxygen saturation of arterial blood during inhalation of air and oxygen. appl. Physiol. *1,* 567, 1949.

ndinav. J. Clin. & Lab. Investigation 297—300, 13, 1961.

THE DISAPPEARANCE OF OXYGEN FROM SHED BLOOD AT BODY TEMPERATURE

By E. ASMUSSEN and M. NIELSEN

From the Laboratory for the Theory of Gymnastics and the Zoophysiological Laboratory, University of Copenhagen, Denmark.

(Received for publication October 27, 1960)

When the previously (Asmussen & Niel- n 1958) described bubble method for deter- nation of arterial oxygen tensions ($P_{a_{O_2}}$) s used at high oxygen tensions (200—300 n Hg) it was found that the systematic rrection for $P_{a_{O_2}}$ was much larger than at rmal or low oxygen tensions. In the latter se it was —0.5 to +1 mm Hg, but at high ygen tensions it was +22 to +27 mm Hg. ie main reason for this large correction is at a certain fall in the oxygen content of e blood during the bubble equilibration ne will cause a much larger decrease in ood oxygen tension because of the almost rizontal shape of the oxygen dissociation rve at these high tensions. This alone, wever, could not explain the large differ- ce. As all precautions were taken to mini- ze the loss of oxygen to the surrounding ids (paraffin oil in the syringes, rinsing id, *etc.*) it seems possible that the dis- pearance of oxygen from blood stored at ° C actually was larger at a high P_{O_2} than lower values of P_{O_2}. For this reason it s decided to study the disappearance of oxygen from blood which was kept anaero- bically at 37° C for different lengths of time. The following conditions were investigated: heparinized blood equilibrated with air mix- tures of a) 5.4 per cent CO_2 and 94.6 per cent O_2, b) 10 per cent CO_2 and 90 per cent O_2, c) 5.4 per cent CO_2 and 15 per cent O_2 in N_2, and d) heparinized venous blood drawn directly from an antecubital vein. Additionally, blood equilibrated with 5.4 per cent CO_2 and 94.6 per cent O_2 was mixed with fluoride (1 mg per ml blood) or sodium cyanide (0.065 mg/ml and 1 mg/ml) and the disappearance of oxygen determined.

METHODS

About 20 ml of freshly drawn venous blood was mixed with a few drops of a heparin solution and placed in a test tube of about 25 ml capacity. A tiny drop of an anti-foam solution was added and the blood was then placed in a thermostated waterbath and bubbled through with the pre-heated, moistened air mixture for about 100 minutes. The blood was now transferred to a sampling tube, previously filled with mercury and also kept in the waterbath. A second, mercury filled, sampling tube was con-

nected to the first through a mercury filled polyethylene tube, avoiding trapping of air in the system. By maneuvering the stopcocks and leveling bulbs of the two sampling tubes the blood could be thoroughly mixed. Samples for analysis were taken by disconnecting the polyethylene tube from the sampling tube and connecting it to a 1 ml Ostwald-pipette. Samples for analysis were taken at intervals through approximately 4 hours. The blood samples were analyzed immediately for CO_2 and O_2 content with the Van Slyke apparatus.

RESULTS

The results from 3 series of experiments in which the blood was equilibrated with air containing 94.6 per cent O_2, 15.6 per cent O_2, or was used directly as venous blood (oxygen content about 6 vol. per cent) ar shown in Fig. 1. Because the scattering o the first analyses usually was a little large than the scattering of the second analyses these latter were used as the starting poin from which the disappearance of oxygen wa calculated. The rate of disappearance wa 0.525 vol. per cent per hour at the hig oxygen content, 0.375 vol. per cent per hou at normal arterial oxygen content and 0.20 vol. per cent per hour in the venous blood

In the series where the CO_2 percent in th equilibration mixture was 10 and the O percent 90, the disappearance rate was 0.55 vol. per cent per hour (Fig. 1, below).

The addition of fluoride (1 mg/ml) o

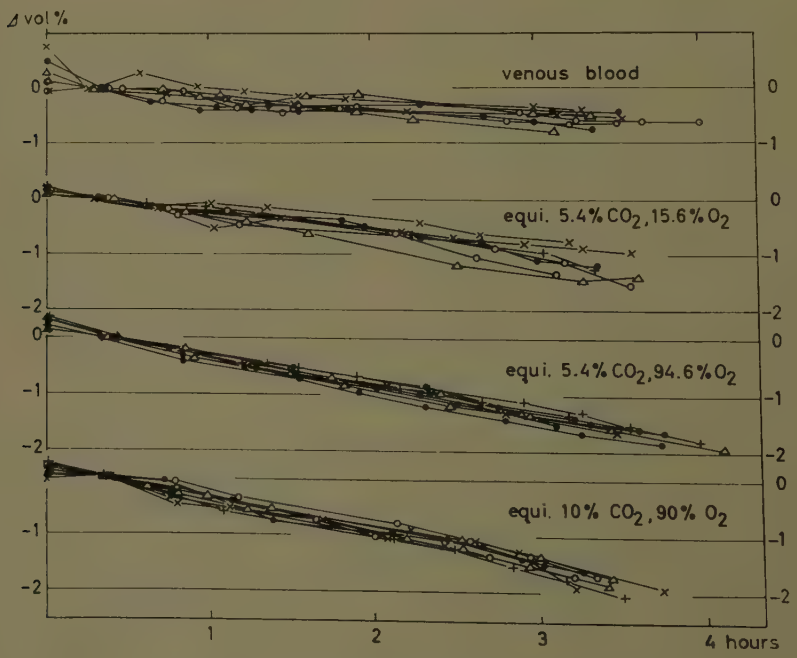


Fig. 1. Fall in oxygen content of blood equilibrated with different air mixtures at 37° C. Signatures mark different subjects.

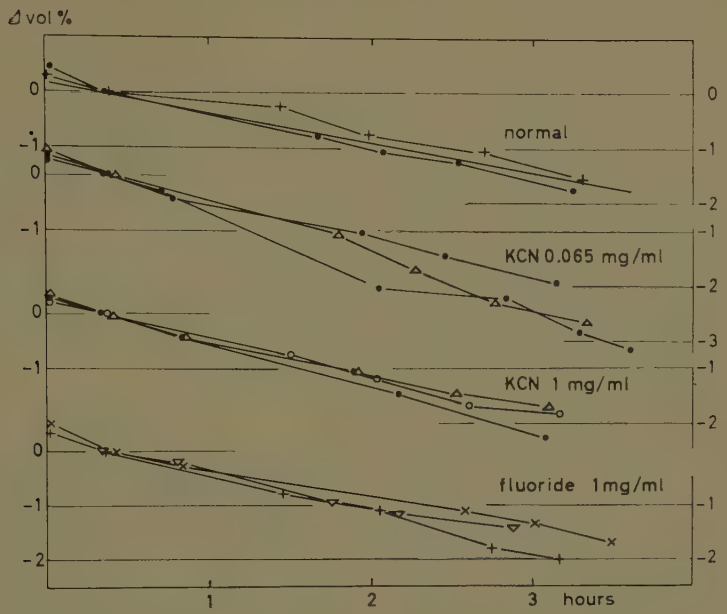


Fig. 2. Fall in oxygen content of blood equilibrated with air mixture of 5.4 per cent CO_2 and 94.6 per cent O_2 with addition of cyanide or fluoride. Signatures mark different subjects.

nide (0.065 mg/ml or 1 mg/ml) had no asurable effect on the rate of disappear-e of oxygen from blood equilibrated with 5 per cent oxygen as is shown in Fig. 2.

COMMENTS

xygen disappears from drawn blood when blood is kept at body temperature. The ner the oxygen tension of the blood, the ner is the rate of disappearance. Partly for reason and partly because of the shape he oxygen dissociation curve for blood, fall in oxygen tension is much larger at high values of blood P_{O_2}. Converted to ion, the disappearance rate would be roximately 175 mm per hour or 3 mm per min. when the Hb is fully saturated *(i. e.* above 400 mm P_{O_2}) but only about 20 mm per hour (0.3 mm Hg per min.) at a blood P_{O_2} of about 100 mm Hg. In venous blood the rate is negligible, only about 1 mm Hg per hour.

It is generally assumed that the disappearance of oxygen from shed blood kept at 37° C is due to oxygen consumption of the blood cells (see Douglas 1909, Berggren 1942). The parallel increase found in CO_2-content of stored blood makes this assumption justifiable. Douglas found that fluoride could delay this metabolism, and cyanide has also been suggested as a means of stopping it. The present experiments confirm, however, the findings of Lambertsen, Bunce,

Drabkin & Schmidt (1952) that neither fluoride nor cyanide has an appreciable effect on the disappearance of oxygen.

The increase in CO_2-content that occurs concomitantly with the fall in oxygen content (Douglas 1909) was also found in the experiments presented here. However, due to the technical difficulties in determining the CO_2-content accurately enough with the Van Slyke method, the precise increase per hour could not be calculated and, therefore the R.Q. of the processes taking place in the blood could not be determined.

The experiments have shown that the disappearance of oxygen from blood stored at body temperature can reach an appreciable size, especially when the Hb is completely saturated. The resulting fall in P_{O_2} is of such a magnitude that special corrections have to be made, for example, when determinations of arterial P_{O_2} are made on shed blood.

SUMMARY

Oxygen disappears from blood stored anaerobically at 37° C. The rate of disappearance was 0.525 vol. per cent per hour at a P_{O_2} above 400 mm Hg, 0.375 vol. p cent per hour at a P_{O_2} around 100 mm H and only 0.200 vol. per cent per hour venous blood. Converted to tension the f would be about 175 mm per hour at hig P_{O_2}, 20 mm per hour at normal arterial P_{O_2} and only about 1 mm Hg per hour at venou P_{O_2}. Addition of fluoride or cyanide had n measurable effect on the rate of disappea ance.

REFERENCES

Asmussen, E. & Nielsen, M.: A bubble method f determination of pCO_2 and pO_2 in bloo Scandinav. J. Clin. & Lab. Investigation 1 267, 1958.

Berggren, S. M.: The oxygen deficit of arteri blood caused by non-ventilating parts of t lung. Acta physiol. Scandinav. *4*, Suppl. 1 1942.

Douglas, C. G.: The oxygen capacity of the blo after hæmorrhage. J. Physiol. *39*, 453, 1909 10.

Lambertsen, C. J., Bunce, P. L., Drabkin, D. & Schmidt, C. F.: Relationship of oxygen te sion to hemoglobin oxygen saturation in t arterial blood of normal men. J. appl. Physi *4*, 873, 1952.

dinav. J. Clin. & Lab. Investigation 301—305, 13, 1961.

RAPID DETERMINATION OF UREA BY A MODIFIED CONWAY TECHNIQUE

By I. HOLM-JENSEN

From the Pharmacological Institute, University of Aarhus, Denmark.

(Received for publication November 21, 1960)

he simple and accurate micro-diffusion cedure for the determination of urea in ll samples of blood developed by Conway 33), Abelin (1938), and Conway & Malley (1942) is used in many clinical oratories.

n this analysis, urea is converted to am- nia by means of urease. By the subse- nt addition of alkali the ammonia is rated and absorbed in a solution of boric , which, after a sufficient lapse of time nearly quantitative transfer to take place, itrated to its initial pH with standard rochloric acid. The duration of this ysis is about 1½ hours, but it can be reciably shortened if stirring is applied ng the diffusion period.

procedure allowing the above analysis e completed in about 30 minutes with about 5 minutes expended on manipula- s in each analysis is described below.

he present modification of the Conway cedure has been worked out with 0.1 ml samples of known solutions of ammonium. sulphate and urea and with blood plasma. The principal features are:

1. The enzymatic conversion of urea into ammonia takes place in a volume of fluid, about 0.125 ml, which is only slightly larger than the volume of the sample.

2. After the completion of the enzymatic reaction 1.0 ml of saturated potassium carbonate is added, and rapid liberation of ammonia occurs due to the solution being nearly 90 per cent saturated with potassium carbonate.

3. Agitation is applied to the Conway units, rendering manual stirring superfluous, and increasing rate of transfer of the ammonia.

4. Magnetic stirring is applied during the titration.

METHOD

List of Laboratory Equipment

1. Modified Conway units (Fig. 1A). In the outer chamber of a Conway standard unit is ground a cylindrical recess, diameter 9 mm, depth 3 mm, the bottom of which must be flat.

2. Special lids for the Conway units (Fig. 1B). The lid is a circular disc made of "Perspex". The diameter, 68 mm, is the same as the outer diameter of the rim of the Conway unit. The lid is provided with a knob, and along the rim of the lower surface there is a circular rabbet, depth about 0.5 mm, to accommodate the upper rim of the Conway unit.

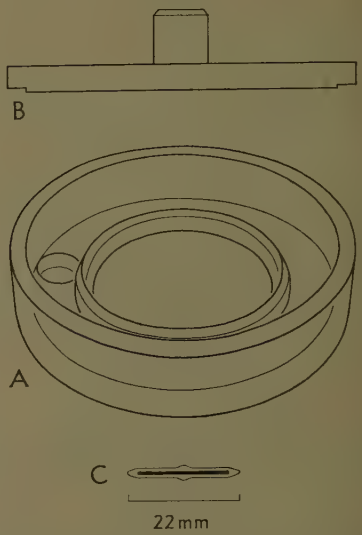
3. Stainless steel balls, diameters 3 mm, for stirring the fluid in the cylindrical recess described above. (Glass beads are not suitable for this purpose).

4. Stirring device, consisting of a sloping turntable (slope 18°, 16 revolutions per minute), as recently described by the author (Holm-Jensen 1960 c) for mixing the sample and reagents and for stirring during the diffusion period.

5. Magnetic stirring device to be used for the titrations. — The arrangement described by the author (Holm-Jensen 1960 b) for use with the Conway No. 2 units is adapted to the Conway standard units by replacing the ring on the roof of the box containing motor and stirring magnet by a larger one. (However, the use of magnetic stirring during the titration is not indispensable in the analysis described.)

6. Stirring bar (Fig. 1C) made of iron wire, 18 x 1 mm enclosed in a glass tube, 22 x 2 mm, the middle of which is slightly bulbous, as shown in the figure.

7. Burette. — The 0.1 ml burette described by the author (Holm-Jensen 1960 a) is very convenient for the titrations.



Modified Conway unit with lid and magnetic st ring bar.

Solutions and Reagents

1. Boric acid reagent with indicator:

Boric acid	g 1
Distilled water	ml 1
0.1 per cent alcoholic solution of bromocresol green	ml 6
0.1 per cent alcoholic solution of methyl red	ml 3

Before use it may be necessary to adjust t solution to the desired end-point colour by the ad tion of a small quantity of alkali.

2. Urease solution. Urease (Sigma, Spe Purity, Type V), 0.1 g is dissolved in 10 ml tilled water. Stored at 0° this solution will k for several weeks.

3. Saturated solution of potassium carbon 115 g potassium carbonate is dissolved in 100 of hot distilled water. At room temperature surplus of a few grammes will precipitate.

4. 100 mN and 33.3 mN standard hydrochl acid for the titrations.

ormance of the Analysis

bout 1 ml of the boric acid reagent is delivered the central chamber of the clean and dry modi- Conway unit described above. A stainless steel is placed in the recess of the outer chamber the sample to be analysed, usually 0.1 ml, is delivered in the recess. The urease solution, lly 25 μl, is added, and the unit is immediately ed with the lid, which has previously been ared with vaseline along the rim. The vessel ow placed on the disc of the stirring device allowed to rotate for at least 15 minutes. Then rotation is stopped; the Conway unit is turned d its axis, so that the recess is in its highest tion. The lid is now displaced, so that a small ing appears into the lower part of the outer nber; 1 ml of the saturated potassium carbonate ickly introduced, and the lid immediately placed osition again. — After another 15 minutes the rotating disc the lid is removed, and the tion carried out without any unnecessary de- — The titration is conveniently performed ng magnetic stirring with the bar described e. The bar is introduced just before the titra- , and the same bar is used for successive titra- s; it can easily be transferred from one Conway to another by means of a clean forceps; no hing between successive titrations is needed. ring is most efficient when the axis of rotation he bar is slightly decentred in relation to the re of the Conway unit.

he urea concentration (in mg/100 ml) in the nl sample is calculated by multiplying the vol- (in μl) of 100 mN acid used in the titration 3.00. When 33.3 mN acid is used, the factor .00.

uring storage the content of ammonia in the se solution increases; therefore blank analyses ld be performed regularly. With the above le of urease, the volume of 33.3 mN standard used in blank analyses was usually about 0.3 μl. With this technique analyses were carried out known solutions of urea ranging from 30 to mg/100 ml. The recovery was about 97 per , and the results were reproducible with a ficient of variation of about 1 per cent.

COMMENTS

Notes on the Method

It is strongly recommended that readers who may decide to make use of the present method should study the monograph of Conway: Microdiffusion Analysis and Volumetric Error (1957) if they are not familiar with this technique.

Below, brief comments are given on some of the principal differences between the present procedure and the methods for the determination of ammonia and urea as described by Conway.

Factors Influencing the Transfer and Recovery of Ammonia

In recovery experiments, samples of 0.1 ml of 50 mM $(NH_4)_2SO_4$ were analysed. The ammonia was liberated by 1 ml of saturated potassium carbonate and absorbed in 1 ml of a solution of 1 per cent of boric acid containing 20 per cent of ethyl alcohol, this composition being recommended by Conway. In these experiments, the length of the diffusion period was 40 minutes, during which time the Conway units were stirred as described; the recovery was found to be 96.8 $\pm$ 0.4 per cent, and it was demonstrated that the recovery could not be increased by using a longer diffusion period. However, it appeared that when the boric acid reagent was replaced by a 1 per cent solution of boric acid in water, the quantity recovered increased to 98.8 $\pm$ 0.3 per cent. The difference is probably caused by the presence of traces of aldehyde in the grades of alcohol commercially available in this country. *It was consequently decided to omit alcohol from the*

boric acid reagent. (The small amount of alcohol present in the indicator solutions proved to be of no significance.)

In the above experiment the approach of an equilibrium with nearly 100 per cent of ammonia absorbed in the boric reagent should be anticipated, so the results are still about 1 per cent too low.

After the uptake of the quantity of ammonia absorbed in the above experiments the boric acid reagent has become alkaline; it was therefore investigated whether the above discrepancy might be caused either by loss of ammonia from the boric acid reagent or by uptake of carbon dioxide from the atmosphere during the titration.

A number of Conway units were exposed to the atmosphere for 60 minutes after completion of the diffusion. The quantity of ammonia recovered by the subsequent titration of the boric acid reagent was now about 85 per cent, and still lower (about 80 per cent) when the boric acid had been stirred during the exposure. In similar experiments, other vessels were exposed during magnetic stirring to a carbon dioxide-free atmosphere, which was passed through holes made in the lids of the Conway units at a rate of about 30 ml per minute. In these experiments, the results of the analyses were nearly the same as in the experiments with exposure to atmospheric air. Hence the principal cause of the apparent loss of ammonia observed must be a real loss of ammonia. In experiments in which the exposure to the air was 4, 8, and 12 minutes, the average loss of ammonia amounted to 0.25 per cent per minute. These findings probably explain why the maximum recovery of ammonia in analyses carried out on 0.1 ml 50 mM $(NH_4)_2SO_4$ was found to be only about 9 per cent.

The loss of ammonia to the atmosphe from boric acid reagent containing one ten of the above quantity of ammonia w negligible (about 1 per cent per hour), a in analyses on 10 µl samples of the 50 m $(NH_4)_2SO_4$ solution the average recove was 99.6 per cent.

The rate of transfer of the ammonia w studied in experiments with 0.1 ml 50 m ammonium sulphate plus 1.00 ml saturat potassium carbonate in the outer chambe The duration of these experiments w varied between 5 and 60 minutes. The resul agreed with calculations based on the a sumption of a constant half life of the a monia in the outer chamber of 2.1 minut

It was also demonstrated that the rate transfer of the ammonia can be further i creased by a simultaneous increase of the t of the turntable (up to 25°) and the rate revolution (up to about 30 r.p.m.), but view of the risk of accidental exchange fluids between the two chambers of the Co way unit thus introduced, this measure ca not be recommended.

Notes on the Enzymatic Conversion of Urea to Ammonia

In the procedures described by Conwa the enzymatic conversion of urea to a monia was accomplished by means of sp cially prepared extracts of jack beans. Ho ever, for the sake of convenience only co mercially available preparations of urea have been considered in the present stu

Among a number of different grades urease tested by the author, Sigma, Spe

ty, Type V, proved to be the best. It
ı high specific activity, and the quanti-
of ammonia originating from the urease
aration are small and independent of the
tity of urea present in the samples ana-
.

ing the technique described above, but
ing 60 minutes for the enzymatic action,
ses were performed on known solutions
ea (Urea *pro analysi,* Merck) contain-
rom 30 to 300 mg per 100 ml. In these
riments the recovery was about 98 per
and it was found that the recovery
not be increased by allowing a longer
d for the enzymatic action. With 15
tes for the enzymatic action the rec-
was determined as 97 per cent, and
10 minutes it was about 95 per cent.
difference could be demonstrated be-
ı urease reagent dissolved in water and
uffer solutions. Very little time was
d by using a more concentrated solu-
of the urease preparation. It was con-
d that the above results also apply to
ses performed on blood plasma.

SUMMARY

modified Conway technique suitable for
etermination of urea in 0.1 ml samples
ood plasma is described. Allowing 15
tes for the enzymatic conversion of urea into ammonia and another 15 minutes for the diffusion process, 97 per cent of the urea content of the samples is recovered with a coefficient of variation of about 1 per cent.

ACKNOWLEDGEMENT

The work was aided by a grant from King Christian X's Foundation.

REFERENCES

Abelin, I.: Über die Mikrobestimmung des Harnstoffs im Blute ohne Destillation und ohne Nesslerisation. Biochem. Ztschr. *297,* 203, 1938.

Conway, E. J.: An absorption apparatus for the microdetermination of certain volatile substances. II. The determination of urea and ammonia in body fluids. Biochem. J. *27,* 430, 1933.

— Microdiffusion analysis and volumetric error, 4th ed. Crosby Lockwood & Son, London 1957.

— & O'Malley, E. O.: Microdiffusion methods. Ammonia and urea using buffered absorbents. (Revised methods for ranges greater than 10 μg N). Biochem. J. *36,* 655, 1942.

Holm-Jensen, I.: A simple and accurate microburette. Scandinav. J. Clin. & Lab. Investigation *12,* 247, 1960, a.

— A modified Conway technique for the determination of carbonates with titration carried out during magnetic stirring in a carbon dioxide-free atmosphere. Scandinav. J. Clin. & Lab. Investigation *12,* 269, 1960, b.

— A simple mechanical stirring device. Its application to rapid determination of carbonates by a modified Conway technique. Scandinav. J. Clin. & Lab. Investigation *13,* 1960, c.

Scandinav. J. Clin. & Lab. Investigation 306—313, 13, 1961.

THE INFLUENCE OF HEPARIN ON THE FIBRIN CLOT

By H. C. GODAL

From the Institute for Thrombosis Research, University Hospital, Rikshospitalet, Oslo, Norway.

(Received for publication November 29, 1960)

There is probably a relationship between the solubility of fibrinogen and the structure and properties of the fibrin clots formed with thrombin. Thus, if the solubility of fibrinogen is reduced by lowering ionic strength, pH, or temperature, or by addition of fibrinogen-precipitating agents such as protamine or dextran, the structure of the clot changes from the "fine", transparent type with little tendency to syneresis to the "coarse", opaque type with greater tendency to syneresis (Ferry & Morrison 1947, Edsall & Lever 1951, Godal 1960 b).

In a previous paper it was shown that small amounts of heparin reduce the solubility of purified fibrinogen; great amounts exert the opposite effect (Godal 1960 a). It is possible, therefore, that heparin, in addition to its antithrombin effect, also influences the polymerization-aggregation process. To solve this problem. I have studied the physical properties of fibrin clots formed in the presence of heparin.

MATERIALS AND METHODS

Heparin, A-L, Oslo, Norway (identical to heparin Vitrum, Stockholm, Sweden), a 5 per cent (w/v) solution, was diluted in saline to the desired strength. The preparation contains 100 I. U. per mg of heparin, or 5,000 I. U. per ml solution.

Since the commercial heparin contains small amounts of tricresol, and phenols influence thrombin-fibrinogen reaction (Fantl & Marr 19 the heparin preparation was dialysed against sa

Otherwise, the materials and methods used the present work have all been described previo (Godal 1960 b).

RESULTS

A. *Studies on purified fibrinogen.*

1) *The effect of heparin on the throm time.* The effect of various concentration heparin on the thrombin time of puri fibrinogen is given in Fig. 1. A weak,

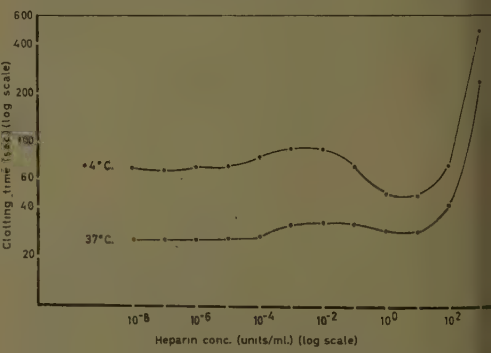


Fig. 1. *The effect of heparin on the thro time of purified fibrinogen.*

Clotting system:
0.2 ml fibrinogen solution (0.3 g per cent)
0.2 ml heparin solution of various concentra
0.2 ml thrombin.
Prior to the addition of thrombin, the fibrino heparin mixtures were incubated for three mi at the temperature chosen for the experime

ificant anticoagulant effect was demonble with very low concentrations of rin. By increasing the heparin concenon, this effect more or less disappeared, it reappeared with large concentrations eparin.

similar correlation between heparin entration and thrombin time has presly been demonstrated by Shinowara 9), by incubation of purified fibrinogen heparin at room temperature before ulation with thrombin. Since heparin is vn to form complexes with thrombin rter & Nanninga 1953, Lyttleton 1954, kwardt & Walzmann 1959), the antiulant effect of small concentrations of rin is probably due to the direct effect eparin without cofactor. However, traces eparin cofactor present in the thrombin ibrinogen preparations cannot be exed.

he anticoagulant effect of large concentrations of heparin was identical to that found in plasma, and will be dealt with below.

2) *The effect of low temperature, low pH, low ionic strength, and calcium on the thrombin time and physical properties of the fibrin clot.* If coagulation occurred at +4° C., a weak clot-promoting effect of medium concentrations of heparin was observed (Fig. 1). A similar effect was observed by Shinowara (1949) in solutions of crude fibrinogen preincubated with heparin at room temperature.

Several observations indicate that this clot-promoting effect is caused by an increased tendency to aggregation of fibrin. Thus, the clots were more coarse and had greater tendency to syneresis than without heparin (Table I). Further, a similar effect on the clot could be obtained by lowering the ionic strength by lowering pH, or by addition of calcium chloride. Finally, the

Table I. *The effect of low temperature, low pH, low ionic strength and of calcium on the thrombin time and syneresis time of heparinized and non-heparinized fibrinogen.*

Experimental condition				Thrombin time (secs.)		Syneresis time (min.)	
pH	Ionic strength	Temperature (° C.)	Calcium added	Heparin	No heparin	Heparin	No heparin
7.3	0.15	37	—	29.6	25.4	13.5	14.5
7.3	0.15	4	—	49.2	69.8	4.5	9.0
7.3	0.10	37	—	16.4	17.2	6.0	12.5
6.5	0.15	37	—	24.9	27.8	6.5	7.5
7.3	0.15	37	+	14.2	15.9	5.5	8.0

Clotting system:

0.2 ml. fibrinogen solution (0.3 g per cent), of various pH, containing 1/5 volume of heparin (50 I.U./ml) or 1/5 volume of psysiological saline,
0.2 ml $CaCl_2$ solution (10 mM) or physiological saline,
0.2 ml thrombin.

Table II. *The relationship between thrombin time and coagulability in heparinized and non-heparinized plasma.*

Heparin		No heparin	
Thrombin time (secs.)	Clottability (per cent of total)	Thrombin time (secs.)	Clottability (per cent of total)
2—3	97		
4.1	88	5.2	101
12.8	48	9.1	98
26.0	26	19.0	100
54.0	14	37.0	86
104.0	3	68.0	53
No clot	No clot	134.0	32

Clotting system:

0.2 ml citrated, platelet poor plasma,
0.2 ml heparin (20 I.U./ml) or physiological saline,
0.2 ml thrombin.

amount of heparin necessary for obtaining maximal aggregation tendency of fibrin is similar to that necessary for obtaining maximal precipitation of fibrin in the cold (Godal 1960 a).

B. *Studies on plasma.*

1) *The relationship between thrombin time and coagulability in heparinized and non-heparinized plasma.* The rapid inactivation of thrombin by heparin + cofactor leads to incomplete coagulation (Burstein 1954, Losner & Volk 1954). Further, the clots are coarse with decreased tensile strength (Tarlow, Goldfarb & Benjamin 1942, Wagreich & Tarlow 1945).

The relationship between thrombin time and the yield of fibrin in the presence of 10 units of heparin per ml plasma is given in Table II. Obviously, the coagulation is incomplete, even with a thrombin concentration which clots the plasma in a few seconds. In contrast, the coagulation of non-hepa nized plasma is complete, even when t thrombin time is 30 seconds. Therefore, ve large concentrations of thrombin are nece sary to produce complete coagulation fibrinogen when relatively large amounts heparin are present. In addition, with lar amounts of thrombin the syneresis time a the tenside strength also tended to becor normal.

2) *The influence of low ionic streng low pH, low temperature, calcium, and de tran on the clots of heparinized plasm* These factors markedly reduce the effect heparin on the thrombin time (Godal 196 They also increase the fibrinogen-precipi ting effect of heparin (Godal 1960 a), this may explain their effect on the throm time.

However, this was apparently not (Table III). In contrast to non-heparini plasma, the clots became finer, with lon

Table III. *The influence of low temperature, low ionic strength, low pH, calcium and dextran on the clottability, tensile strength and syneresis time of heparinized and non-heparinized plasma.*

Experimental condition				Clottability (per cent of total)		Tensile strength (g)		Syneresis time (min)	
Ionic strength	Tempera-ture (° C.)	$CaCl_2$	Dex-tran	Heparin	No heparin	Heparin	No heparin	Heparin	No heparin
0.15	37	—	—	15	98	17	96	2.5	14.0
0.15	20	—	—	51	101	46	83	9.0	11.5
0.10	37	—	—	38	102	29	94	8.0	10.5
0.15	37	—	—	31	96	37	90	6.0	9.5
0.15	37	+	—	28	104	27	117	5.5	10.5
0.15	37	—	+	34	98	—	—	6.5	9.0

Clotting system:

0.2 ml citrated, platelet-poor plasma of various pH, containing 1/10 volume of heparin (100 I.U./ml) or 1/10 volume of physiological saline,
0.2 ml distilled water $CaCl_2$ (10 mM), dextran (3 g per cent) or physiological saline,
0.2 ml of thrombin solution.

When tensile strength was assayed, 1.0 ml of plasma, 0.6 ml of distilled water, $CaCl_2$, dextran or saline, and 0.2 ml of a stronger thrombin solution were used.

resis times. In addition, the coagula- y and tensile strength increased. The cts on the fibrin clots are similar to those d when the thrombin concentration is eased (see Table II), and they are there- mainly due to a reduced antithrombin ct of heparin and not to an increased ipitating effect of this compound.

The effect on the fibrin clot of high entrations of heparin. The inhibitory t of high concentrations of heparin on thrombin-fibrinogen reaction differed in ral respects from that observed with ler concentrations. It could be demon- ed in solutions of purified fibrinogen . 1), and was thus independent of hepa- cofactor. Further, the clots formed were less opaque and more rigid than with- heparin, with longer syneresis times (Table IV). The observations suggest an inhibitory effect on the polymerization-aggregation process, and are in concordance with the observation that high concentrations of heparin increase the solubility of fibrinogen (Godal 1960 a).

It is interesting to note that, in the presence of high concentrations of heparin, the effect of neutral salts on the thrombin time differs from that which is seen with lower concentrations, and also from that which is seen in non-heparinized plasma. Thus, in the presence of sufficient amounts of sodium chloride, a clot-promoting effect of high concentrations of heparin could be demonstrated (Fig. 2). No explanation for this phenomenon can be given at present, but as judged from the properties of the clot (Table IV), it seems that by increasing the salt concentration, the effect of high concentrations of

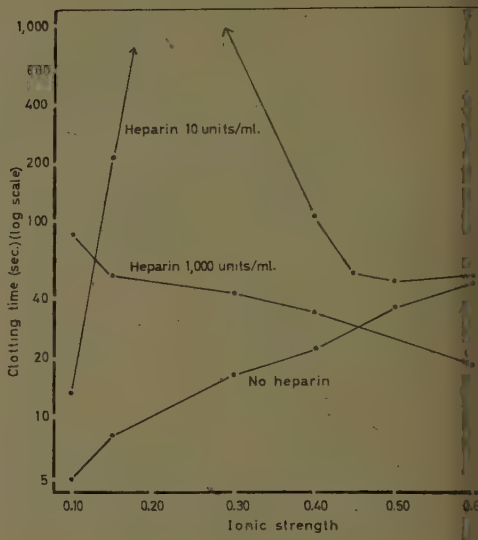


Fig. 2.

The effect of neutral salt on the thrombin time of plasma in the presence of various concentrations of heparin.

Clotting system:

0.2 ml citrated, platelet-poor plasma, containing 1/5 volume of heparin (5,000 I.U./ml, or 50 I.U./ml), or 1/5 volume of physiological saline.

0.2 ml distilled water, or NaCl solutions of various strengths

0.2 ml thrombin.

The clotting mixtures were incubated at 37° C for three minutes prior to the addition of thrombin.

heparin on the polymerization-aggregation of fibrin, changes from inhibitory to accelerator.

4) *The effect of protamine on the clot of heparinized plasma.* As shown previously, protamine shortens the thrombin time of non-heparinized plasma, probably by affe ting the polymerization-aggregation of fibr (Godal 1960 b).

The effect of increasing concentrations protamine on the thrombin time and on t properties of the fibrin clot in the presen of 10 units of heparin per ml plasma,

Table IV. *The effect of neutral salt on the clotting time and syneresis time of plasma containing large amounts of heparin (1,000 units per ml), as compared with non-heparinized plasma.*

Ionic strenght	Clotting time (secs.)		Syneresis time (min,)	
	Heparin	No heparin	Heparin	No heparin
0.10	42	Instan.	>120	11
0.60	9	39	41	>120

Clotting system:

0.2 ml citrated, platelet-poor plasma, containing 1/5 volume of undiluted, dialysed heparin, or 1/5 volume of physiological saline,

0.2 ml distilled water, or 0.2 ml of NaCl solution (1.5 M),

0.2 ml of thrombin solution.

Table V. *The effect of various concentrations of protamine on thrombin time, clottability, tensile strength and syneresis time of heparinized plasma.*

Protamine µg/ml.	Thrombin time (secs.)	Clottability (per cent)	Tensile strength (g)	Syneresis time (min.)
0	No clot	—	—	—
2.5	186	12	25	1.5
5.0	38	47	34	6.0
10.0	21	94	84	13.0
No protamine No heparin	22	101	81	14.5

Clotting system:

0.2 ml citrated, platelet poor plasma, containing 1/10 volume of heparin (5 I.U./ml) or 1/10 volume of physiological saline,

0.2 ml of protamine solution of various strengths, or 0.2 ml of physiological saline,

When tensile strength was assayed, 0.8 ml of plasma, 0.8 ml of protamine or saline, and 0.2 ml of a stronger thrombin were used.

n in Table V. It is seen that parallel to normalization of the thrombin time, the itself will also be normalized. This ob- ation might be useful in order to see ther or not a clotpromoting effect of heparin agents is specific.

DISCUSSION

inowara (1949) observed that on in- tion of purified fibrinogen with heparin om temperature, the thrombin time de- ed progressively and finally became shorter than without heparin. The or assumed that a factor, called P, was onsible for this clot-promoting effect. factor, more abundant in highly puri- fibrinogen than in crude preparations, l be removed by adsorbtion with barium nate or by Seitz filtration. However, according to the observations made above, this clot-promoting effect of heparin is most likely due to precipitation of fibrinogen. This view is supported by the fact that the fibrinogen solutions used by Shinowara were opalescent before, but not after adsorbtion or filtration, and further, incipient precipitation occurred prior to the addition of thrombin.

In contrast, the experiments with plasma demonstrated that precipitation was of little importance for the antiheparin effect of such factors as low temperature, low ionic strength, low pH, or the presence of calcium or dextran. Burstein & Guinand (1955), claimed that the clot-promoting effect of calcium on heparinized plasma was not accompanied by an increase in fibrin yield. The data given above are contradictionary to those given by Burstein *et al.*, and this discrepancy cannot be explained. However,

since not only calcium, but also the other factors studied in this work all proved to increase the fibrin yield, it is reasonable to believe that the antithrombin effect of heparin is influenced in some way by the speed of the polymerization-aggregation process. The following tentative explanation of this relationship is suggested. Heparin is capable of complexing several plasma proteins, and the stability of these complexes increases when the proteins are denatured (Fischer 1935). Thus, if heparinized plasma or purified fibrinogen is clotted with thrombin, or the fibrinogen precipitated by heat, the concentration of heparin in the supernatant will decrease, indicating that a part of the heparin coprecipitates with fibrin(ogen) (Godal, unpublished data). When thrombin is added, heparin will be bound to polymerized fibrin, and less heparin will be available for the inactivation of thrombin. If the polymerization-aggregation process for some reason is speeded up, this will counteract the antithrombin effect of heparin.

SUMMARY

The effect of heparin on the properties of the fibrin clot has been studied with the following results:

1) Under certain circumstances, heparin shortens the thrombin time of highly purified fibrinogen, probably due to precipitation of fibrin(ogen) by heparin.

2) An inverse relationship between the tendency to aggregation of fibrin and the antithrombin effect of heparin seems to exist. This relationship is probably due to a competition between fibrin(ogen) and heparin cofactor-thrombin for heparin.

3) High concentrations of heparin se to counteract the polymerization-aggregati process. This effect can be reversed by creasing the ionic strength. In the prese of sufficient amounts of neutral salt, a cl promoting effect of such concentrations heparin can be demonstrated.

4) Protamine, in concentrations just s ficient to normalize the prolonged throm time of heparinized plasma, also produce normalization of the properties of the fib clot. This observation might be useful order to see whether or not a clot-promot effect of an antiheparin agent is specific.

REFERENCES

Burstein, M.: Sur l'effect antagoniste de l'hépar au cours de la deuxième phase de la coagu tion. Semaine d. hôp. Paris *30*, 3051, 1954

Burstein, M. & Guinand, A.: Sur le temps thrombine du plasma héparine. Rev. d'hém *10*, 567, 1955.

Edsall, J. T. & Lever, W. F.: Effects of ions neutral molecules on fibrin clotting. J. B Chem. *191*, 735, 1951.

Fantl, P. & Marr, A. G. M.: Phenols as inhibit of the heparin co-factor of plasma. Nature 990, 1957.

Ferry, J. D. & Morrison, P. R.: Preparation properties of serum and plasma proteins. V The conversion of human fibrinogen to fil under various conditions. J. Am. Chem. Soc. 388, 1947.

Fischer, A.: Die Bindung von Heparin an Eiwe Biochem. Ztschr. *273*, 133, 1935.

Godal, H. C.: Precipitation of human fibrino with heparin. Scandinav. J. Clin. & L Investigation *12*, 56, 1960 (a).

— The interaction of protamine with hu fibrinogen and the significance of this i action for the coagulation of fibrinogen. *12*, 433, 1960 (b).

— The assay of heparin in thrombin syst Ibid. 1961 (in press).

ter, E. & Nanninga, L.: Complexes of heparin and proteins. Faraday Soc. Disc. 1953, no. 13, p. 205.

ner, S. & Volk, B. W.: Fibrinogen preservation in serum after heparin. Proc. Soc. Exper. Biol. & Med. *54,* 417, 1954.

tleton, J. M.: The antithrombin activity of heparin. Biochem. J. *58,* 15, 1954.

rkwardt, F. & Walzmann, P.: Untersuchungen über den Mechanismus der Antithrombinwirkung des Heparins. Ztschr. f. physiol. Chem. *317,* 64, 1959.

Shinowara, G. Y.: Enzyme studies on human blood. IV. Interrelation of heparin and fibrinogen fractions. Am. J. Physiol. *156,* 458, 1949.

Tarlow, I. M., Goldfarb, A. J., & Benjamin, B.: A method for measuring the tensible strength and stretch of plasma clots. J. Lab. & Clin. Med. *27,* 1333, 1942.

Wagreich, H. & Tarlow, J. M.: Studies on the strength of fibrinogen thrombin clots. Arch. Biochem. *7,* 345, 1945.

Scandinav. J. Clin. & Lab. Investigation 314—325, 13, 1961.

HEPARIN TOLERANCE AND THE PLASMA PROTEINS

By H. C. GODAL

From the Institute for Thrombosis Research, University Hospital, Rikshospitalet, Oslo, Norway.

(Received for publication November 29, 1960)

Increased heparin tolerance, as measured by various *in vitro* techniques, has repeatedly been observed in patients with, or predisposed to, thrombosis (for references, see Gormsen 1957). As a rule, the increased heparin tolerance has been taken to reflect an increased coagulability. Recently, Holger-Madsen (1959) demonstrated that the recalcification time and the thrombin time of heparinized plasma (HTT) both varied in parallel during the course of myocardial infarction or after major operations (Holger-Madsen & Schiøler 1960), indicating that identical factors are responsible for the increased heparin tolerance in both clotting systems.

In a previous paper, I have found that the heparin thrombin time of citrated plasma is influenced by a great number of factors, among them the concentrations of various plasma protein fractions (Godal 1961). Since a number of plasma protein fractions are known to increase during inflammatory and degenerative diseases, it is possible that such protein fractions, at least in part, are responsible for the increased heparin tolerance measured by HTT. The purpose of this paper was to investigate this possibility.

Fibrinogen and alpha$_1$-glucoprotein were found to be important for the short HTT found in patients suffering from various diseases. The glucoprotein fraction itself, a weak anticoagulant (Greenspan 1951), proved to be an effective inhibitor of heparin, probably by competition.

MATERIALS AND METHODS

Alpha$_1$-glucoproteins. a) Isolation. The procedure of Weimer, Mehl, & Winzler (1950) was followed, starting from platelet-poor, citrated plasma. The lyophilized product was a white powder which dissolved easily in water to a faintly turbid solution. The preparation was nearly homogeneous on paper electrophoresis (pH 8.6), travelling as an alpha globulin. It did not precipitate on boiling. The lyophilized material was dissolved in physiological saline to desired concentration (see below).

b) Determination was performed according to the method of Winzler, Devor, Mehl & Smyth (1948), except that the protein content of the phosphotungstic acid precipitate was determined by reading the extinction at 280 mμ, after dissolution in 3 ml of 1/5 saturated sodium carbonate with the solvent as a blank. A 0.05 per cent solution of purified plasma albumin, treated in the same way, was used as a standard.

Cholesterol in serum was determined according to the method of Carr & Drekter (1956). The

yses were carried out by the Central Labora-
, Rikshospitalet, Oslo. Normal values: 150—
mg/100 ml.

Dextran sulphate. Dexulate, "Glaxo", Green-
, England was diluted in saline to the desired
ngth. It is the sodium salt of dextran sulphate,
an average molecular weight of 7,500. It
ains 25 units per mg of dextran sulphate, or
0 units per ml solution.

Fibrinogen. A commercial preparation from
bi", Stockholm, Sweden, containing about 95
cent clottable protein, was used. The lyophili-
material was dissolved in distilled water to a
ein concentration of 2 per cent, and the solution
ysed against physiological saline at pH 7.3 for
hours at + 4° C. Insoluble material was re-
ed by centrifugation at 3,000 r.p.m. for half
iour. The final solution was slightly opalescent.

Haptoglobin in serum was determined by the
ition procedure described by Nyman (1959).
analyses were kindly carried out by Dr. T.
mark.

Hemoglobin. Human erythrocytes were washed
e times in saline and hemolysed by freezing
thawing. After centrifugation, the clear super-
nt was dialysed for 24 hours against physio-
cal saline at + 4° C. The hemoglobin content
he final solution was determined spectrophoto-
rically at 540 mμ, according to Donaldson,
on, King, Wotton & Macfarlane (1951). A
k solution, containing 5 per cent hemoglobin
stored at —20° C, and diluted with saline to
red strength after thawing.

Heparin A-L, Oslo, Norway (identical to hepa-
"Vitrum", Stockholm, Sweden) was used. The
tion contains 5 per cent heparin, and 100 I.U.
ml in saline was stored at —20° C. After
wing, this solution was further diluted in saline
he desired strength. The same stock solution
used for all the experiments.

Heparin cofactor was determined in the fol-
ing clotting system:

0.2 ml fibrinogen solution, 0.3 per cent,
0.2 ml test plasma, diluted 1:9 with saline, or 0.2 ml of various dilutions of standard plasma,
0.1 ml heparin solution (0.30 I.U. per ml), or saline,
0.1 ml thrombin solution, adjusted to give a clotting time of about 25 seconds with standard plasma diluted 1:9 with saline, and with saline instead of heparin in the test system.

The clotting mixture was incubated for exactly three minutes at 37° C prior to the addition of thrombin. The amount of heparin used prolonged the clotting time of the test system with standard plasma diluted 1:9 from 25 to about 55 seconds. A standard curve was made by testing various dilutions of standard plasma in this system.

Heparin thrombin time (HTT). When nothing else is stated, the following clotting system was used:
0.2 ml fresh, platelet poor, citrated plasma,
0.2 ml heparin solution (0.15 I.U./ml),
0.2 ml thrombin solution.

The clotting mixtures were incubated at 37° C for exactly three minutes prior to the addition of thrombin. Freshly prepared thrombin solution (see below) was titrated in a preliminary experiment to clot the standard plasma in about 13 seconds without heparin and in about 95—115 seconds with heparin present. This concentration of heparin was found to be suitable, since a marked anticoagulant effect is evident, and yet the clotting time is not too difficult to read. With higher heparin concentrations, as used by Holger-Madsen, I found it very difficult to determine the clotting time accurately.

The coagulation procedure had to be strictly standardized, and the following technique was followed: After addition of heparin, the test tube was *immediately* shaken, and thereafter tilted every 15 seconds. The appearance of a clot was taken as end point. The test was routinely run in triplicate, the first serving as a guide. The mean of the last two tubes was recorded.

Storage of plasma. In accordance with the observations of Holger-Madsen (1959), it was neces-

sary to centrifuge the blood within half an hour after withdraval, to avoid shortening of HTT. *After* centrifugation, more than one hour was necessary to alter the plasma, even when stored at room temperature. The plasmas were always tested within one hour after centrifugation. A single freezing and thawing did not alter the plasmas appreciably, and in most cases, storage at —20° C for a few days had no effect. When stored for weeks, however, a shortening of HTT invariably occurred, especially in the plasmas from the patients. Therefore, fresh plasma was always used for the test.

Effect of citrate concentration. It is well known that citrate enhances the anticoagulant effect of heparin. In the clotting system used here, a citrate concentration equivalent to that of plasma from citrated blood, with a hematocrit of 25, shortened the HTT from about 100 seconds to about 70 seconds. In this work, the citrate concentration was corrected for by diluting the plasma with citrate when the hematocrit was below 35.

Paper electrophoresis of serum proteins was carried out by the Central Laboratory, Rikshospitalet, Oslo. An Elphor apparatus (Bender & Hobein, Munich, Germany) with a veronal (Michaelis) buffer of pH 8.6, ionic strength 0.10 was used. The samples were run for 18 hours at 90 volt on Schleicher & Schuell No. 2043 A filter paper strips, stained with Amido black, and read in a Spinco Analytrol Scanner. A globulin factor of 1.14 was used.

Platelet-poor, citrated plasma. The blood was collected through a siliconized needle of wide bore directly into a lusteroid tube containing 1/10 volume of 3.13 g per cent sodium citrate dihydrate, placed on melting ice, and centrifuged within half an hour at 3,000 r.p.m. for 30 minutes at + 4° C. For other purposes than determination of HTT, the plasma was stored at —20° C.

Thrombin, "Topostasine", Hoffmann-La Roche, Basel, Switzerland, a bovine preparation (powder) was used. Each day an amount (usually one mg) of the thrombin powder was dissolved in saline, and adjusted to the desired strength by testing th activity on standard plasma. During the clottin experiments, the thrombin solution was kept a room temperature. For all experiments the sam batch of thrombin powder was used.

Total serum protein was determined by the Biu ret method. The analyses were carried out by th Central Laboratory, Rikshospitalet, Oslo. Norma values are given in Table II.

Plasma containing various concentrations fibrinogen was prepared in the following way Plasma fraction I was precipitated with eight pe cent ethanol, according to Cohn, Strong, Hughe Mulford, Ashworth, Melin & Taylor (1946). Afte centrifugation, the supernatant was immediatel lyophilized in order to remove the ethanol, an reconstituted to its original volume by distille water, and pH adjusted to 7.3 with 1/15 N HC This defibrinated plasma was then mixed wit various dilutions of purified fibrinogen in proportio 2:1.

Otherwise, the methods and materials used this work have been described previously (God 1960 b).

RESULTS

Heparin thrombin time (HTT) in norm individuals. Ten healthy individuals, 4 me and 6 women, age 20—40 years, were teste on the same day. HTT varied from 80 to 1 seconds with a mean of 109.9 seconds. Pla ma from the donor, used as standard durin this work, had a HTT of 98 seconds.

HTT in patients. The aim of this wo has been to study possible correlations b tween various plasma protein fractions an HTT. Therefore, patients with various a normal protein patterns were selected for t study, and no attention paid to the unde lying disease. The patient material, co prizing 43 patients, is thus very heteroge

(see Table I), and a short HTT was
ıd under various pathological conditions.
small number of patients does not per-
conclusions as to correlation between
ous pathological conditions and a short
T, but the data suggest that a short HTT
ht be observed more frequently in pati-
with collagen diseases, renal diseases,
anced diabetes mellitus, Hodgkin's dis-
, leukemia and cancer, than in peptic
r, hepatitis, hemolytic anemia and hemo-
ia.

he plasma proteins in patients with short T. Total serum protein, serum protein tionation by paper electrophoresis, plas- fibrinogen and total serum cholesterol indicator of beta lipoproteins) were de- ined in patients with HTT below 80 nds and compared with corresponding es of healthy individuals. (The normal es for total serum protein, electro- retic fractions and cholesterol are those n by the Central Laboratory, Rikshospi- , as based on large series of determina- s. The normal fibrinogen values are d on the normals used as controls in this k.) The patient with myeloma of the beta ulin type (K.S.) has been excluded. The for this patient are given below.

he result is given in Table II. As would expected, there are marked individual ations. Generally, a low albumin, in- sed alpha- and gamma globulin, and eased fibrinogen were found. Such pro- patterns are generally found in acute chronic active, inflammatory and dege- tive diseases, and are thus unspecific. most marked deviation from normal was d in the alpha globulin fractions and in nogen.

Table I. *HTT in 43 patients suffering from various diseases.*

Pat.	Age	Sex	Diagnosis	HTT
T.M.	15	F	Lupus eryth. nodos	23
C.F.	54	F	Collagen disease	29
A.B.	23	F	Arteritis	69
Å.Å.	70	F	Polyarthritis chron.	91
O.H.	62	M	Colitis	62
I.T.	25	M	Hodgkin's disease	36
B.A.	22	M	» »	42
G.S.	19	F	» »	40
A.S.	72	M	» »	51
A.Ø.	21	F	» »	61
T.N.	19	F	Granulomatosis	38
O.G.	67	F	Leukemia	27
J.K.	35	M	»	57
P.B.	58	F	»	63
R.B.	78	M	»	51
K.S.	59	M	Myeloma	44
R.H.	48	M	»	105
K.F.	58	M	»	210
E.O.	56	M	Ca. ventriculi	56
P.H.	63	M	» »	76
J.E.	43	M	Cancer?	52
E.D.	25	F	Diabetes mellitus	28
S.R.	54	F	» »	64
A.M.	48	M	» »	58
B.L.	51	F	» »	39
S.Ø.	27	F	» »	34
O.T.	32	F	» »	74
M.M.	49	F	Pyelonephritis	30
G.W.	63	F	Chron. nephritis	35
G.C.	45	F	Ren. cysticus	46
A.J.	47	M	Hepatitis acuta	120
P.B.	61	M	» chron?	54
E.G.	17	F	» chron.	135
M.E.	38	F	» »	95
V.R.	58	F	Cholelithiasis	98
Å.N.	34	F	Ulcus ventriculi	125
A.B.	46	F	» »	108
R.A.	42	F	Hemolyt. anemia	111
K.A.	29	M	Parox. noct. hematuria	121
O.S.	34	M	Hemophilia A	81
B.E.	63	M	» »	104
T.H.	55	M	» B	165
M.B.	46	F	Tot. proacc. def.	113

The importance of various plasma protein fractions for a short HTT.

1) *Albumin.* A weak antiheparin effect of purified albumin could be demonstrated in normal plasma (Table III), in agreement

Table II. *Various plasma protein fractions in 28 patients with HTT shorter than 80 seconds; as compared with corresponding normal values (in g per cent).*

	Patients		Normals	
	Mean	Range	Mean	Range
Total serum Protein	6.9	5.3—7.9	7.1	6.5—7.5
Albumin	3.2	1.8—4.1	4.3	3.6—4.9
α_1—globulin	0.45	0.3—0.6	0.3	0.2—0.4
α_2—globulin	0.77	0.4—1.3	0.45	0.3—0.6
β—globulin	1.0	0.7—1.2	0.9	0.6—1.2
γ—globulin	1.65	0.8—3.8	1.2	0.9—1.6
Fibrinogen	0.547	0.255—0.805	0 264	0.238—0.310
Cholesterol	0.279	0.166—0.376	—	0.150—0.300

with earlier observations (Godal 1961). Whether this effect is due to albumin itself or to contaminants, cannot be stated at present. It is therefore possible that low plasma albumin (as found in the patients) reduc the heparin tolerance as measured by HT

Table III. *Effect of various protein fraction on HTT of normal plasma.*

Protein fraction tested (final concentration 2 g per cent).	HTT
Albumin	67
Gamma globulin ...	81
Macroglobulin	44
Hemoglobin	28
Control (saline)	104

Clotting system:
- 0.2 ml platelet-poor, citrated plasma,
- 0.1 ml protein solution (4 per cent), or physiological saline,
- 0.1 ml heparin (0.30 I.U./ml),
- 0.2 ml thrombin.

2) *Alpha$_1$-glucoproteins.* Among thes the fraction studied here (MP-1 of Mel Humphrey & Winzler 1949, alpha$_1$-ac glucoprotein of Schmid 1955) is marked increased in cancer and pyogenic infectio (Winzler & Smyth 1948). In several r spects, this fraction is similar to heparin. has a very low isoelectric point (1.8) a high contents of sulphur and carbohydra (Weimer, Mehl & Winzler 1950). It is weak anticoagulant (Greenspan 1951, Nil son & Yamashina 1958) and the antico gulant effect can be neutralized by protami or toluidine blue (Greenspan 1951). Nothi is known about the effect of this protein the presence of heparin.

In the patients studied here, a rough co relation between the level of alpha$_1$-gluc protein and HTT was observed (Fig. Further, isolated alpha$_1$-glucoprotein inh bited the antithrombin effect of hepa (Fig. 2). A final concentration of 0.6—

00 ml plasma was sufficient to neutralize anticoagulant effect of 0.15 I.U. of hepaper ml plasma, and concentrations similar those found in the patients definitely rtened the HTT. It is therefore likely t the increased concentration of alpha$_1$-coprotein contributes to the short HTT nd in the patients.

Several observations suggest that the coprotein fraction competes with heparin. ce this fraction has no anticoagulant effect lf when measured in a thrombin system ilsson & Wenckert 1958), such a comtion must lead to shorter HTT. Further, ther heparin-like substance, dextran sulte, which is only poorly active in the tem used (Hjort & Stormorken 1957), ved to inhibit the antithrombin effect of arin, when mixed in proper proportions g. 3). Finally, the unspecific "fibrinostic" effect of the glucoprotein fraction much weaker, and the antiheparin effect ch stronger than that of a typical "fibrinostic" substance, dextran (Table IV).

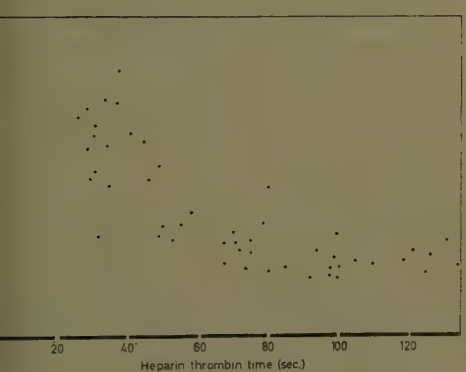


Fig. 1.
relation between HTT and plasma content of alpha$_1$-glucoprotein.

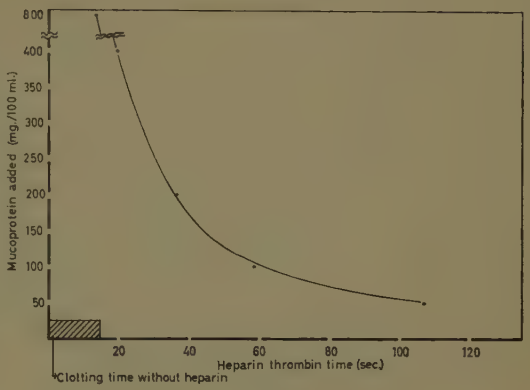


Fig. 2.
Fffect of purified alpha$_1$-glucoprotein on HTT of normal plasma.

Clotting systems: 0.2 ml platelet-poor, citrated plasma,
0.1 ml glucoprotein solution,
0.1 ml heparin (0.30 I.U/ml),
0.2 ml thrombin.

3) *Haptoglobins (hp)* are a group of closely related seromucoids with alpha$_2$-mobility at pH 8.6 (barbiturate buffer). Their concentration in plasma varies grossly parallel to that of the alpha$_1$-glucoprotein during inflammatory and neoplastic diseases (Nyman 1959). With hemoglobin, hp form very stable complexes, reacting in stoichiometric (equimolecular) proportions (Jayle 1940). Since the formation of such complexes may be inhibited by heparin (Nyman 1959), hp probably counteract the antithrombin effect of heparin.

However, such an effect could not be demonstrated. Thus, addition of hemoglobin to give a final concentration equimolecular to that of hp, did not prolong the HTT even in patients with very high hp content in plasma. On the other hand, excess hemoglobin definitely shortened HTT (Table III).

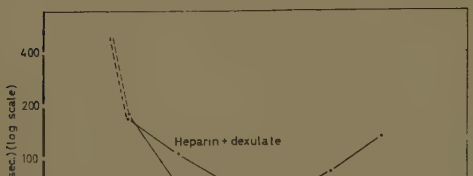


Fig. 3.

Effect of various concentrations of dextran sulphate (dexulate) on HTT of normal plasma.

Clotting systems: 0.2 ml platelet-poor, citrated plasma,
0.1 ml of various dilutions of dexulate,
0.1 ml heparin (0.30 I.U/ml),
0.2 ml thrombin.

Since purified haptoglobins were not available, a direct proof of the effect on HTT of hp has not been given. Further work is therefore necessary. However, the result of this investigation seems to indicate that hp are of little importance for HTT.

4) *Fibrinogen.* Addition of purified fibrinogen to citrated plasma shortens the thrombin time in the presence of larger amounts of heparin (Godal 1961). Fibrinogen might therefore also be affective in the system used here.

Generally, the patients with short HTT had increased levels of fibrinogen. Further, if fibrinogen concentration was plotted against HTT, a rough correlation was demonstrable (Fig. 4). Finally, normal plasma containing various amounts of fibrinogen (see methods) demonstrated a similar correlation (Fig. 4). These findings suggest that the fibrinogen concentration is of importance for HTT.

Is plasma fraction I in the patients qualitatively different from normal? Plasma fraction I (I) and supernatant (S) were prepared as described (see methods) from normal plasma. The fractions were mixed in various ways and the HTT of the mixtures recorded. The result is given in Table V. (I) from the patients + normal (S) gave shorter HTT than of normal (I) + normal (S), even when the higher fibrinogen content in the patients (I) had been corrected for. In addition, (S) from the patients shortened the HTT of normal (I).

The first observation suggests that plasma fraction I from the patients studied differed qualitatively from that of normals. It cannot

Table IV. *Effect of dextran and alpha$_1$-glucoprotein on the thrombin time of normal plasma with and without heparin present.*

Material tested (final concentration 1.5 g per cent).	Thrombin time (sec)	
	With heparin	Without heparin
Dextran.....	18.4	7.1
α_1-glucoprotein...	12.3	11.1
Control (saline) ..	89	13.1

Clotting system:
0.2 ml platelet-poor, citrated plasma,
0.1 ml dextran or glucoprotein solution (3 per cent) or physiological saline,
0.1 ml heparin (0.30 I.U./ml) or saline,
0.2 ml thrombin.

tated whether this difference is due to a
itatively different fibrinogen, or whether
s due to other proteins, coprecipitated
fibrinogen in plasma fraction I. The
nd observation strengthens the evidence
ght forward above, that factors other
those contained in plasma fraction I are
nportance for HTT.

Beta lipoproteins. Heparin is known

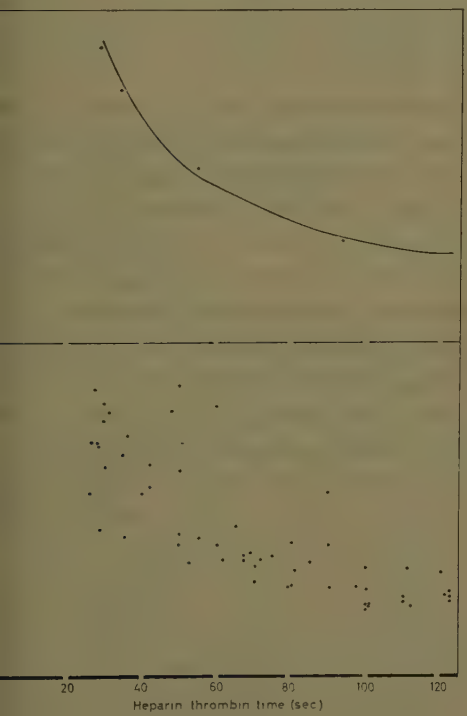


Fig. 4.

Fibrinogen concentration and HTT.
e: HTT of normal plasma containing various amounts of purified fibrinogen.
ing systems: (for details, see methods).
0.2 ml supernatant,
0.1 ml fibrinogen solution,
0.1 ml heparin (0.30 I.U./ml),
0.2 ml thrombin.
w: Correlation between HTT and plasma content of fibrinogen.

Table V. *(For details, see text.)*

Experimental conditions	HTT	
	Pat. T. M.	Pat. C. F.
Normal (S) + Normal (I).....	116	116
» » + Pat. »	25	36
Pat. » + Normal »	27	40
» » + Pat. »	17.5	25
Normal » + Pat (I) (corr.).. ..	68	74
Pat. » + » » »	26	38

Clotting system:
0.2 ml plasma mixture,
0.2 ml heparin (0.15 I.U./ml),
0.2 ml thrombin.

to form complexes with the beta lipoproteins (Burstein 1956). Beta lipoproteins might therefore interfere with HTT.

However, most of the patients studied had normal serum levels of beta lipoproteins, as judged from their serum cholesterol levels (Table II). To study a probable effect of beta lipoproteins, two groups of patients were selected, both consisting of patients suffering from angina pectoris with no recent or actual episodes of myocardial infarction or thrombosis. In group I, the serum cholesterol was normal or slightly elevated, in group II it was markedly elevated. Both groups were on long-term treatment with Phenylindandione (Trombantin, Nyco, Oslo, Norway). Both groups had a moderately shortened HTT (Table VI) without any difference between the groups. The reason for the shortened HTT in these patients is not known. They had, however, higher plasma levels of both fibrinogen and alpha$_1$-glucoprotein than had the normal controls. Further work is necessary to prove whether these differences are due to age, to treatment with Phenylindondione or to the disease itself.

Table VI. *HTT, fibrinogen, alpha$_1$-glucoprotein and cholesterol in patients suffering from angina pectoris. (For details, see text.)*

Number	I	II	Normals
	10	5	10
HTT (sec)	Mean; 71.2 (59—83)	74.6 (56—100)	109.9 (78—148)
Fibrinogen (mg/100 ml.)..	Mean: 338 (272—491)	318 (266—438)	264 (238—310)
α_1-glucoprotein (mg/100 ml.)..	Mean: 84 (76—100)	86 (77—93)	63 (55—74)
Cholesterol (mg/100 ml.)..	Mean: 354 (288—396)	523 (406—628)	— —

6) *Gamma globulin.* No correlation between gamma globulin content and HTT could be demonstrated. Further, patients with markedly increased gamma globulin levels, such as in hepatitis, had normal HTT (Table I). Finally, purified gamma globulin had only a very slight antiheparin effect in the clotting system used here (Table IV). Therefore, gamma globulins probably are not important for HTT.

7) *Paraproteins.* One patients (R.H.) with myeloma of the gamma globulin type had normal HTT in spite of a concentration of gamma globulin of 5.6 g/100 ml. Another patient (K.F.) with only minor electrophoretic disturbance had a markedly prolonged HTT. The reason for this is not known; he had normal values of alpha$_1$-glucoprotein, fibrinogen and heparin cofactor.

In contrast, a patient (K.S.) with myeloma of the beta globulin type had markedly shortened HTT. His serum was very viscous at low temperature, and proved on ultracentrifugal analysis (Carlsberg Foundation, Copenhagen, Dr. Djurtoft), to contain three abnormal fractions with sedimentation constants of 10.2, 8.9, and 7.3. These fractions constituted about 3 g per cent of prote (total serum protein: 9.4 g per cent, a total beta globulins: 6.2 g per cent). It possible that the shortened HTT in t patient is due the content of "macroglob lins", since isolated macroglobulins fr another patient studied previously (Go 1961) proved to shorten the HTT of norm plasma (Table III).

8) *The clotting factors.* Heparin inhib both the formation and the action of thro bin. Further, the content of antihemophi globulin (AHG) increases roughly paral with fibrinogen in the diseases studied h (Egeberg, personal communication). T clotting factors may therefore influe HTT.

However, removal of prothrombin, p convertin, Christmas factor (PTC) a

Table VI. *Heparin cofactor content in patients with HTT shorter than 80 seconds as compared with normal values.*

	27 Patients	10 Normals
Heparin cofactor (per cent)	Mean: 94 (48—160)	Mean: 103 (85—130)

art factor by adsorbtion of citrated plas- with $Al(OH)_3$ did not affect the HTT atients or in normals. Further, patients total deficiency of AHG, PTC or pro- lerin had normal HTT (Table I). ally, activation of plasma by SiO_2 lite) did not shorten HTT. Therefore, T seems to be independent of the plasma tent of clotting factors (except fibri- en).

) *Heparin cofactor (Hcf).* For the anti- mbin effect of heparin, a plasmatic co- or is necessary, and the thrombin time of arinized plasma is very sensitive to varia- s in Hcf content (Godal 1961). A short T might therefore be due to a low con- of this factor.

Iowever, patients with short HTT had ghly normal contents of Hcf, as deter- ed by the method described above ble VII). Further, no correlation be- en Hcf and HTT could be demonstrated. 'ariation in Hcf, therefore, seems to be minor importance for the HTT in the ents studied here.

DISCUSSION

t is well established that heparin tolerance ncreased in many patients. This findings nterpreted to indicate an increased coagu- lity, and this "hypercoagulability" is eved to be an important factor in the hanism of thrombosis. However, there several weak points in this line of oning.

Thus, there are so far no studies on the nges in plasma which lead to an in- sed heparin tolerance. We do not know ch factors are responsible for it, nor do know whether the same foctors are changed in all patients. Further, we are not justified in interpreting increased heparin tolerance in terms of hypercoagulability, since we know nothing about the role of heparin in these patients. It is perfectly possible that the changes which produce an increased heparin tolerance may at the same time inhibit coagulation *in vivo*. Finally, we are not justified in interpreting increased heparin tolerance in terms of increased tendency to thrombosis for the same reasons.

In this situation, the first step must be to study the protein changes which constitute the basis for the increased heparin tolerance, and this has been the aim of the present study.

The data reported indicate that the concentration of various clotting factors (except fibrinogen) does not influence the heparin tolerance when measured in a thrombin system. Since such factors influence the heparin tolerance when other techniques are used (recalcification time, whole blood clotting time), the thrombin system should be preferred when proteins other than clotting factors are studied.

Among the protein fractions studied here, the $alpha_1$-glucoprotein fraction probably deserves special attention. Evidence has been brought forward here that this protein fraction competes with heparin. Since high concentrations of $alpha_1$-glucoproteins are rather closely related to increased heparin tolerance, this might also explan why plasmas with increased heparin tolerance also have increased protamine tolerance (Weiner & Jiminez 1956). Further, the fact that $alpha_1$-glucoproteins are increased under circumstances where thrombosis is more likely to occur, this observation makes the

importance of this heparin-like substance as a physiological anticoagulant rather doubtful. It is possible that the glucoprotein fraction, in addition to its weak anticoagulant effect, also has unfavorable effects on the coagulation and hemostasis mechanisms from the point of view of preventing thrombosis. It is interesting to note that dextran sulphate and other similar substances produce clumping of platelets and precipitation of fibrinogen (Piper 1946, Astrup 1953, Walton 1954). In fact, the glucoprotein fraction studied here had a weak "fibrinoplastic" effect. Further work along these lines, therefore, is necessary. Another problem which has not been taken into consideration, is the question whether or not the glucoproteins are also qualitatively altered during disease. Further work to study these problems is in progress.

The antiheparin effect of purified fibrinogen is in concordance with earlier observations (Godal 1961). However, fibrinogen is easily denatured, and denatured fibrinogen (similar to other denatured proteins) interacts more readily with heparin (Fischer 1935, Godal 1960 a). Purified fibrinogen, therefore, cannot be compared with the native protein as to its antiheparin effect. Another possibility is that fibrinogen becomes altered during disease, and that such altered fibrinogen more easily interacts with heparin. Smith (1957) observed that fibrinogen from patients suffering from various diseases precipitated more easily with heparin in the cold than did fibrinogen from healthy individuals, and this difference could not be explained solely as a result of higher fibrinogen contents in the patients. These observations have been confirmed (Godal, unpublished data), and they may furthe explain why plasma fraction I in the patien was more effective in inhibiting hepari However, proteins other than fibrinogen a present in plasma fraction I, and the con position of these proteins during disea might well differ from normal. Therefore, cannot be stated at present whether or n the antiheparin effect of plasma fraction in the patients is due to qualitatively altere fibrinogen.

SUMMARY

The importance of some plasma protei fractions for the increased heparin toleran observed in patients suffering from variou diseases has been studied, giving the follow ing results:

1) In concentrations similar to tho found in the patients, alpha$_1$-glucoprotei and fibrinogen definitely counteract the ant thrombin effect of heparin.

2) 0.6—0.8 g/100 ml of alpha$_1$-glucopr tein fraction completely neutralizes the ant thrombin effect of 0.15 I.U. of hepari Evidence has been brought forward that competitive relationship exists between hep rin and the glucoprotein fraction.

3) Purified fibrinogen inhibits the an coagulant effect of heparin. Plasma fraction from the patients was more effective th that from normal individuals in this respe even when corrected for the higher fib nogen content.

4) The remaining protein fractions stu ied (albumin, haptoglobins, beta lipoprote gamma globulin, paraproteins, clotting fa tors and heparin cofactor), proved to be only minor importance for the increas heparin tolerance observed in the patien

ept that macroglobulins seem to coun-
ct heparin.

REFERENCES

up, T.: The toxicity of synthetic heparin sub-
stitutes. Scandinav. J. Clin. & Lab. Investiga-
tion *5,* 137, 1953.

stein, M. & Samaille, J.: Nouvelle methode de
séparation et de dosage des lipoprotéins de
faible densité. Ann. Biol. Clin. *17,* 23, 1959.

, J. J. & Drekter, I. J.: Simplified rapid
technic for the extraction and determination
of serum cholesterol without saponification.
Clin. Chem. *2,* 353, 1956.

n, E. J., Strong, L. E., Hughes, W. L. Jr.,
Mulford, D. J., Ashworth, J. N., Melin, H.
& Taylor, H. L.: Preparation and properties
of serum and plasma proteins. J. Am. Chem.
Soc. *68,* 459, 1946.

aldson, R., Sisson, R. B., King, E. J., Wotton,
I. D. P. & Macfarlane, R. G.: Determination
of haemoglobin. VII Standardised optical data
for absolute estimations. Lancet, *I,* 874, 1951.

berg, O.: Personal communication.

her, A.: Die Bindung von Heparin an Eiweiss.
Biochem. Ztschr. *278,* 133, 1935.

al, H. C.: Precipitation of human fibrinogen
with heparin. Scandinav. J. Clin. & Lab. Inves-
tigation *12,* 56, 1960, a).

Interaction of protamine with human fibrino-
gen and the significance of this interaction for
the coagulation of fibrinogen with thrombin.
Ibid. *12,* 433, 1960, b).

The assay of heparin in thrombin systems.
Ibid. *13,* 153, 1961.

msen, J.: Koagulationsproblemer ved acut coro-
narthrombose. Thesis. Copenhagen 1957.

enspan, E. M.: The heparinoid nature of a
serum mucoprotein. Science, *114,* 395, 1951.

rt, P. & Stormorken, H.: A study of the in
vitro and in vivo effect of a synthetic heparin-
like anticoagulant: Dextran sulphate. Scandi-
nav. J. Clin. & Lab. Investigation *9,* Suppl. 29,
1957.

ger-Madsen, T.: The plasma heparin thrombin
time. Acta Haemat. *21,* 301, 1959.

Heparin resistance in acute coronary occlu-
sion measured by the heparin thrombin time.
Ibid. *23,* 195, 1960.

— & Schiøler, M.: Increased heparin resistance
after operations measured by the heparin throm-
bin time. Acta chir. Scandinav. *118,* 257, 1960.

Jayle, M. F.: Evaluation molèculaire de l'hapto-
globine par une réaction enzymatique de pero-
xydation. C. R. Acad. Sci. *211,* 574, 1940.

Mehl, J. W., Humphrey, J. & Winzler, R. J.:
Mucoproteins of human plasma; electrophore-
tic studies of mucoproteins from perchloric
acid filtrates of plasma. Proc. Soc. Exper.
Biol. & Med. *72,* 106, 1949.

Nilsson, I. M. & Yamashina, J.: Effect of human
α_1-acid glycoproteins on blood coagulation.
Nature, *181,* 711, 1958.

Nyman, M.: Serum haptoglobin. Methodological
and clinical studies. Scandinav. J. Clin. & Lab.
Investigation *11,* Suppl. 39 1959.

Piper, J.: Toxicology of synthetic polysaccharide
polysulphuric acid esters. Acta Pharmacol. *2,*
317, 1946.

Schmid, K.: Isolation of a group of α_2-glycopro-
teins from human plasma. J. Am. Chem. Soc.
77, 742' 1955.

Smith, R. T.: A heparin-precipitable fraction of
human plasma. II Occurrence and significance
of the fraction in normal individuals and in
various disease states. J. Clin. Investigation *36,*
605, 1957.

Walton, K. W.: Investigation of the toxicity of
series of dextran sulphates of varying mole-
cular weight. Brit. J. Pharmacol. *9,* 1, 1954.

Weiner, M. & Jiminez, M.: Significance of clot-
ting tests augmented by heparin. Am. J. Clin.
Path. *26,* 243, 1956.

Weimer, H. E., Mehl, J. W. & Winzler, R. J.:
Studies on the mucoproteins of human plasma.
V. Isolation and characterization of a homo-
geneous mucoprotein J. Biol. Chem. *185,* 561,
1950.

Winzler, R. J., Devor, A. W., Mehl, J. W. &
Smyth, I. M.: Studies on the mucoproteins of
human plasma. I Determination and isolation.
J. Clin. Investigation *27,* 609, 1948.

— & Smyth, I. M.: Studies on mucoproteins of
human plasma. Plasma mucoprotein levels in
cancer patients. Ibid. *27,* 617, 1948.

Scandinav. J. Clin. & Lab. Investigation 326—331, 13, 1961.

SERUM CREATININE DETERMINATION USING AN ION EXCHANGE RESIN

By A.-C. TEGER-NILSSON

From the Chemical Central Laboratory Sabbatsbergs Sjukhus, Stockholm, Sweden.

(Received for publication November 25, 1960)

Serum creatinine concentration and endogenous creatinine clearance furnish valuable information about renal function. However, the low serum concentration and the non-specificity of most reactions yet tested, has hampered the routine clinical application of creatinine determination.

Several color reactions for creatinine have been described but the Jaffe reaction, which depends on the colored products obtained from creatinine and picric acid in alkaline solutions, is the most widely used in spite of its non-specificity. The compounds in serum interfering with the Jaffe reaction, which are sometimes called "chromogenes" or "pseudocreatinine", include among others glucose, acetone and acetoacetic acid.

Various attemps to eliminate the effect of the "chromogenes" have been described. Miller & Dubos (1937) estimated the "true" creatinine concentration from the difference between the values obtained using the Jaffe reaction before and after the enzymatic destruction of creatinine. Taussky (1956) removed interfering compounds by oxidizing with hypoiodite and extracting the oxidation products with ether. Other authors (Bo sook (1936), Hare (1950), Haugen & Bl gen (1953), Paget, Goutier & Liefoogl (1956), Kayser & Molitor (1956)) ha adsorbed the creatinine from a deproteiniz serum onto different adsorbents and th applied the Jaffe reaction to the creatini after desorption.

In the present investigation the creatini was adsorbed directly from acidified seru on a strong cation-exchanger. This remov most interfering compounds and the Jaf reaction can be applied after elution of t creatinine. The analyses were done wi micro ion exchange columns. As these a rather simple to handle the method may suitable for routine clinical use.

METHOD

Apparatus.

Ion exchange columns were prepared with Dow 50 W, grain size 200—400 mesh as shown in Fig. Glass wool with a fiber diameter of 0.01—0.02 m or cotton wool plugs were used. When carefu packed such a column will not run dry for at le 12 hours because of the capillary forces in

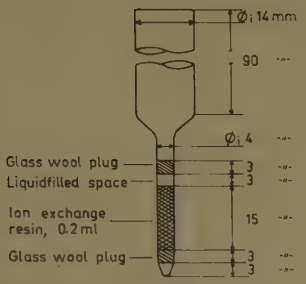


Fig. 1.

exchange column for serum creatinine determination.

wool plug. When stored for a longer period olumns were placed in distilled water.
Radiometer *G* 200 *B* glass electrode was used H measurements.

ents.

M HCl.
3 mM *PICRIC ACID SOLUTION*, (11.75 l).
LKALINE PICRATE SOLUTION, pH 12.4. 0 ml 51.3 mM picric acid solution add 0.80 ml M NaOH. (Should be freshly prepared.)
HOSPHATE BUFFER, pH 12.4. Mix 50 ml M Na_2HPO_4 and 63 ml 0.50 M NaOH and with distilled water to 200 ml.

REATININE STANDARD SOLUTIONS. creatinine (*Ph.S.* XI) to constant weight 0°. From a stock solution containing 1 mg/ml nine in 0.1 M HCl, prepare standard solutions ining 30—300 μg/ml by dilution with distilled .

dure.

Mix equal volumes of serum and 0.1 M HCl small flask and leave it for 2—3 min. to let n dioxide escape. Pour 2.0—4.0 ml of this re on the column of ion exchanger (in H^+-. The flow rate should be about 1 drop per e.

2) Wash the column with 3 ml 0.9 per cent NaCl, and then with 5—6 ml distilled water.

3) Elute the column with 2.0 ml phosphate buffer solution, and collect the eluate in a test tube containing 1.0 ml of alkaline picrate solution. This elutes all the creatinine and the Jaffe reaction takes place in the test tube. Between the different steps of adsorption, washing and elution the column must drain until no more liquid spontaneously leaves the tip.

4) 30 minutes after completed elution measure the absorbance at 510 mμ in a 1 cm cell (Beckman DU spectrophotometer slit width 0.02, or Beckman B spectrophotometer) against a blank consisting of 2.0 ml phosphate buffer solution and 1.0 ml alkaline picrate solution.

Estimate the creatinine from a standard curve, which is obtained by measuring the absorbance of mixtures of 0.1 ml creatinine standard solutions 30—300 μg/ml with 1.9 ml phosphate buffer solution and 1.0 ml alkaline picrate solution.

To check the quality of the ion exchanger, run standard solutions through the columns, and then follow the procedure described for the serum samples. The standard curves obtained with and without passage through the ion exchanger should agree within 3 per cent.

After use the ion exchanger can be regenerated by washing with 2 ml 2—2.5 M NaOH, 1—2 ml distilled water, 2—3 ml 4 M HCl and finally washing with water (approximately 5 ml) until the effluent is neutral.

CONTROL OF THE DIFFERENT STEPS OF DETERMINATION

Adsorption procedure.

Due to its basicity creatinine is adsorbed on strong cation exchangers from an acidic medium and is desorbed in alkaline solution. Dowex 50 was chosen because of its current use in amino acid chromatography. In later experiment Dowex 50 W proved to give superior results. Columns were prepared according to Fig. 1. They do not run dry for

at least 12 hours and thus watching is not necessary. A cycle of adsorption, washing, elution, regeneration and washing requires 6—8 hours, but the actual effort needed during this time is very little.

In spite of the acidic medium the serum proteins do not precipitate in the resin bed and a preliminary protein precipitation therefore seems to be unnecessary. Probably small amounts of serum proteins are irreversibly adsorbed and may finally give rise to errors. The columns can, however, be used for at least ten cycles without renewing the ion exchanger.

The evolution of carbon dioxide when the serum comes in contact with the acidic ion exchanger may ruin the uniform packing of the column. To prevent this the serum is diluted with hydrochloric acid and left for a few minutes before it is passed through the column.

After the adsorption of the creatinine and other cations, a small but varying amount of the ion exchanger is present in the hydrogen form. Before elution it is desirable to convert this part to the sodium form. This is conveniently done by washing with a small volume of dilute sodium chloride solution. Larger volumes (more than 6 ml 0.9 per cent saline) will elute creatinine.

Elution procedure.

For elution various buffers were investigated. 0.125 M phosphate buffer seems to eluate creatinine quantitatively in a small volume. Fig. 2 shows an example of the elution curve obtained with this buffer. When the serum creatinine concentration is very high some tailing occasionally occurs but this has not amounted to more than 2 per cent

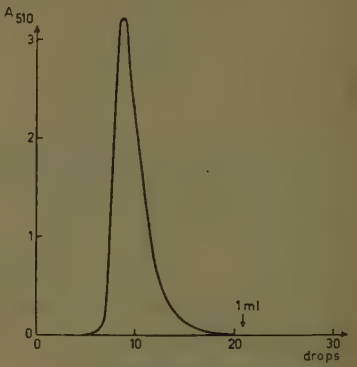


Fig. 2.

Elution curve.

Sample: 2 ml serum with a creatinine concentration of 15 mg/100 ml.

Eluting buffer: 0.125 M phosphate buffer pH 12

Ion exchanger: Dowex 50, 300—400 mesh.

of the total creatinine. A fine grain size the ion exchange resin and a low flow ra are most important for good results in t elution step.

The Jaffe reaction.

It is desirable to have an eluate on whi the Jaffe reaction can be done directly wi out any adjustment of pH or volume. A cording to previous authors the reaction pr ceeds in alkaline medium, but the alkalin used has varied; (Bonsnes & Tauss (1945), Roscoe (1953) and Løken (1954

To find the optimum conditions for method described here, the Jaffe reaction w studied in phosphate buffer solutions different pH. Fig. 3 shows the effect of ti and pH on the absorbance obtained. T color development at pH 10—11 contin for several hours but the absorbance ne reaches the value obtained at pH 12.5. T absorbance at pH 13 reached its high value rapidly and then slowly decreased d

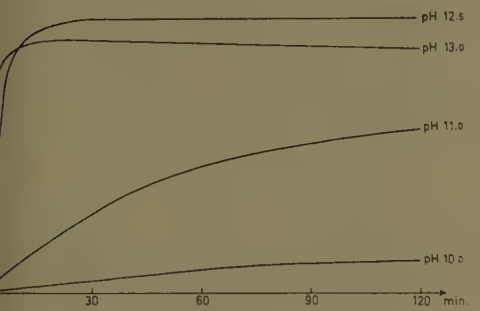


Fig. 3.

or development in the Jaffé reaction as a function of time at different pH values.

the whole observation time, 22 hours. At 12.5 the color development is rather id and the maximum absorbance is high stable for several hours. Accordingly a fer in this region was selected for elution. As stated by previous authors, the tempe- ure affects the Jaffe reaction. According Løken (1954) the differences in optical sity are negligible at room temperature an alkalinity of 0.04 N NaOH. As these ditions closely correspond to the con- ons used here the effect of temperature not re-examined.

The standard curve for the spectrophoto- ric determination in a 0.125 M phosphate fer of pH 12.4 is a straight line in the ion of 3—30 µg of creatinine per 3 ml of l solution. The curve runs independently the buffer concentration within a wide ge (0.06—0.25 M) and consequently the tion of the buffer, which takes place dur- the elution, will not affect the result. As buffer also is well suited for the elution, her volume nor pH of the eluate need be usted and the Jaffe reaction can be carried immediately.

Interfering substances.

A major problem in the determination of the endogenous serum creatinine by the Jaffe reaction is the presence of interfering compounds, which tend to give too high absorbance values in the direct determination. The most important interfering compounds, glucose, acetone, aceto-acetic acid, and pyruvic acid, are not adsorbed on the ion exchanger and their effect is therefore eliminated by the present procedure.

Basic compounds will, however, be found together with creatinine in the eluate. The influence of some amino acids and nitrogenous metabolites on the Jaffe reaction was therefore examined.

The following amino acids were investigated: glycine, alanine, leucine, threonine, phenylalanine, tyrosine, tryptophane, cystine, methionine, proline, hydroxyproline, histidine, arginine, citrulline, glutamine, glutamic acid, valine, cysteine, lysine, isoleucine. None of these seems to interfere with the color reaction up to concentrations corresponding to 50 mg/100 ml of serum, which is well above the normal serum concentration range. Xanthine, hypoxanthine, adenine, uracil, guanine, cytidine, uric acid, urea, choline, *p*-aminohippuric acid, barbituric acid, all in a concentration corresponding to ten times the conceivable serum concentration, have no effect on the Jaffe reaction. Not even guanidine, guanido acetic acid or creatine interfere in spite of their structual resemblance to creatinine.

Hydantoin and allantoin give a color reaction with alkaline picrate. However, these substances when added to serum in a concentration of 10 mg/100 ml do not give any interference in the final reaction mixture when

creatinine is determined by the method described.

Reproducibility.

The recovery of creatinine from standard solutions and from serum samples was investigated. With the use of Dowex 50 the recovery from standard solutions was slightly over 100 per cent, which was probably due to small amounts of chromogen material given off from the ion exchanger. Using Dowex 50 W no such background was found and the recovery approaches 100 per cent. The recovery from serum is indicated in Tables I and II.

By means of a series of double determinations on serum from normal persons the standard deviation for the method was calculated. It was 0.22 mg/100 ml or 2.7 per cent of the mean value.

Normal values.

With the present method the mean value for 30 men was 0.91 mg/100 ml serum with a standard deviation of 0.12 mg/100 ml and for 22 women 0.69 mg/100 ml with a standard deviation of 0.08 mg/100 ml. The normal values seem to agree with norm values found using other other methods su posed to be specific (Miller & Dubos 193 Haugen & Blegen 1953, Taussky 1956 a Edwards & Whyte 1959). The difference serum creatinine concentration for men a women is significant ($P < 0.001$).

Table I. *Recovery of creatinine added to serum.*

Creatinine added µg	Creatinine found µg	Recovery %
—	17.55	—
19.39	19.56	101
21.14	21.52	102
23.10	23.30	101
24.61	25.05	102
26.88	26.71	99
28.84	28.30	98
30.05	29.83	99
31.26	31.29	100
34.73	34.04	98

Table II. *Ten analyses of the same serum using different serum volumes.*

Ml serum taken for analysis	Creatinine found mg/100 ml
0.5	0.78
0.6	0.75
0.7	0.80
0.8	0.76
0.9	0.77
1.0	0.78
1.25	0.79
1.5	0.78
1.75	0.79
2.0	0.77

SUMMARY

A method for serum creatinine determin tion is described. Creatinine is adsorbed fro acidified serum on a strong cation exchan resin in micro columns, eluted with a alkaline buffer solution and determined b the Jaffe reaction performed directly on t eluate. The method seems to give the tr serum creatinine concentration and is simp enough for routine clinical work.

REFERENCES

Bonsnes, R. W. & Taussky, H. H.: On the colo metric determination of creatinine by the Ja reaction. J. Biol. Chem. *158,* 581, 1945.

Borsook, H.: Micromethods for determination ammonia, urea, total nitrogen, uric acid, cre tinine (and creatine), and allatoin. J. Bi Chem. *110,* 481, 1935.

ırds, K. D. G. & Whyte, H. M.: Plasma crea-
inine level and creatinine clearence as tests of
enal function. Australasian Ann. Med. *8,* 218,
959.

, R. S.: Endogenous creatinine in serum and
ırine. Proc. Soc. Exper. Biol. & Med. *74,*
48, 1950.

gen, N. H. & Blegen, E. M.: The true endo-
genous creatinine clearance. Scandinav. J. Clin.
& Lab. Investigation *5,* 67, 1953.

er, F. & Molitor, A.: Emploi de la Mont-
norillonite pour dosage de la creatinine dans
e serum sanguin. Ann. Biol. Clin. *14,* 223, 1956.

n, F.: On the determination of creatinine in
plasma by the Jaffe reaction, after adsorption
to Lloyd's reagent. Scandinav. J. Clin. & Lab.
Investigation *6,* 325. 1954.

Miller, B. F. & Dubos, R.: Determination by a specific, enzymatic method of the creatinine content of blood and urine from normal and nephritic individuals. J. Biol. Chem. *121,* 457, 1937.

Paget, M., Gontier, M. & Liefooghe, J.: Rechearches sur le dosage de la créatininémie II. Ann. Biol. Clin. *14,* 229, 1956.

Roscoe, M. H.: The estimation of creatinine in serum. J. Clin. Path. *6,* 201, 1953.

Taussky, H.: A procedure increasing the specifity of the Jaffe reaction for the determination of creatine in urine and plasma. Clin. chim. Acta *1,* 210, 1956

Scandinav. J. Clin. & Lab. Investigation 332—336, 13, 1961.

SIMULTANEOUS DETERMINATION OF CYCLOSERINE AND PYRAZINAMIDE IN BLOOD SERUM [1]

By K. B. BJÖRNESJÖ

From the Department of Clinical Chemistry, Central County Hospital of Karlstad, Sweden.

(Received for publication November 18, 1960

In chemotherapy of tuberculosis there are individual variations in the absorption of various tuberculostatic drugs from the intestine. Reliable methods for the determination of such drugs are therefore needed to control that an effective blood concentration is obtained. In 1956 Jones published a method for the determination of cycloserine in blood serum with sodium nitritopenta-cyanoferroate. In the presence of cycloserine in weak acid solution this reagent gives a blue reaction product, with an absorption maximum at 625 mμ. When using this method it was found that certain sera from tuberculous patients gave a red discoloration of the reaction mixture on addition of the cycloserine reagent. We observed that these sera originated from patients treated with both cycloserine and pyrazinamide. It was this observation that led to a study of the reaction between pyrazinamide and the cycloserine reagent.

[1] Lecture held at the Scandinavian Congress on Clinical Chemistry in Helsingör 1959.

EXPERIMENTAL

Pyrazinamide was found to give a red color w sodium nitritopentacyanoferroate, with an abso tion maximum at 490 mμ. This reaction ob Lambert-Beer's law and can thus be used quantitative determination of pyrazinamide. In cess of the reagent the reaction occurs independen of the reaction with cycloserine; and the absorpt maxima given of pyrazinamide and cycloserine situated so far from each other that the two re tions do not interfere with each other (See Fig. Consequently the reaction with sodium nitritopen cyanoferroate can be utilized for the simultane determination of cycloserine and pyrazinamide blood serum.

The relation between time and color developm has been studied at various temperatures. Fig thows the reaction between the sodium nitritoper cyanoferroate reagent, cycloserine and pyrazi mide at 20° C. The color produced by the pyrazi mide is not stable at this temperature. At 10° however, the reaction products of both cycloser and pyrazinamide are stable for a period from to 60 minutes (Fig. 3). In accordance with th observations Jones' original method has been m fied for the simultaneous determination of pyraz mide and cycloserine.

Reagents:

1. Sodium nitritopentacyanoferroate reag $Na_4Fe(CN)_5NO_2$. This reagent is prepared im

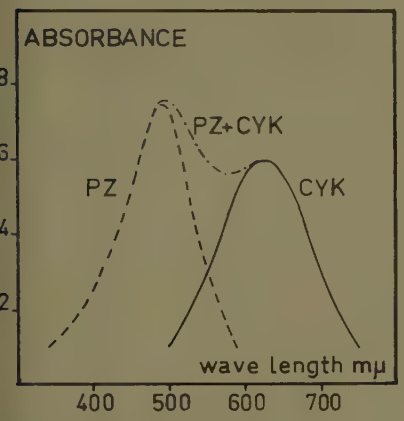


Fig. 1.

ption spectra of the reaction products between sodium nitritopentacyanoferroate reagent, cycloserine, and pyrazinamide.
Absorption spectrum of a solution containing 10 mg pyrazinamide per 100 ml.
= Absorption spectrum of a solution containing 10 mg cycloserine per 100 ml.
- CYK = Absorption spectrum of a solution ning 10 mg pyrazinamide and 10 mg cycloserine per 100 ml.

y before use by mixing aliquots of 4 per cent sodium nitroprusside, $Na_2Fe(CN)_5NO.2H_2O$, N sodium hydroxide. (The sodium nitroide solution can be kept for two weeks at

ungstic acid reagent, This is prepared daily ixing aliquots of 10 per cent w/v sodium ate, Na_2WO_4, and 0.67 N sulfuric acid. N acetic acid.

d:

e serum proteins are precipitated by mixing erum with 4 ml tungstic acid reagent in 15 ml fuge tubes. The tubes are centrifuged at 2500 for 10 minutes. Three ml aliquots of the natant fluids, corresponding to 1 ml serum, ansferred to new tubes. Then 1 ml 3 N acetic added to each tube, and the tubes are cooled C in a bath with running tap water. One ld sodium nitritopentacyanoferroate reagent is added to each tube; the contents are carefully mixed, and the tubes are kept in running tap water at 10° C for 20 minutes. The samples are read in a spectrophotometer at 490 and 625 mμ against a blank of 3 ml water, 1 ml 3 N acetic acid and 1 ml sodium nitritopentacyanoferroate reagent. The cycloserine and pyrazinamide contents are calculated from standard graphs. The standard graph for cycloserine is prepared by analyzing given amounts of cycloserine dissolved in serum, and that for pyrazinamide, by analyzing standard solutions of pyrazinamide in water. Cycloserine and pyrazinamide standards are run parallel in each series.

Accuracy:

The standard error of a single determination (δ_i) has been calculated from the deviations of double determinations according to the formula:

$$\delta_i = \pm\sqrt{\frac{\Sigma d^2}{2N}}, \text{ (Dahlberg 1948)}$$

(d = difference, N = number of differences)

For cycloserine it is 0.10 and for pyrazinamide 0.12 mg in the range of 2.5—10 mg per 100 ml serum.

Recovery experiments:

In a series of experiments various amounts of cycloserine and pyrazinamide were dissolved in serum, and these solutions were compared with standard solutions containing a corresponding amount of cycloserine and pyrazinamide in water. The results are shown in Table I. The mean recovery of pyrazinamide is 98.5 per cent, whereas that of cycloserine is only 76.5 per cent. Further experiments have indicated that the loss of cycloserine is caused partly by a co-precipitation of cycloserine with the serum proteins, and partly by a slow destruction of cycloserine by

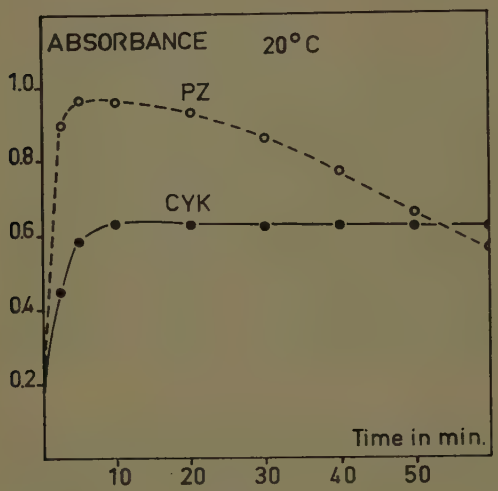


Fig. 2.

The reaction between the sodium nitritopentacyanoferroate reagent, pyrazinamide and cycloserine at 20° C. Relation between time and color development. Pyrazinamide is read at 490 mμ, and cycloserine at 625 mμ.

PZ = 1 mM solution of pyrazinamide.
CYK = 1 mM solution of cycloserine.

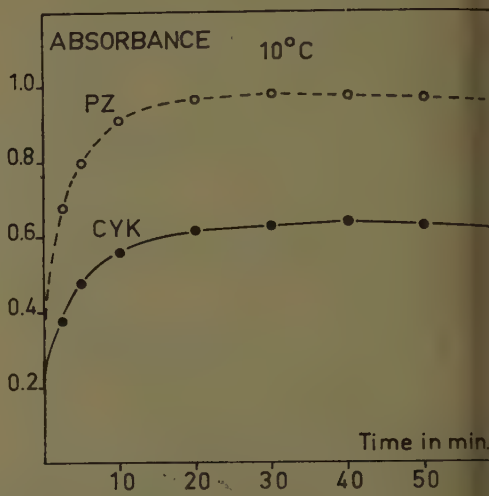


Fig. 3.

The reaction between the sodium nitritopentacya ferroate reagent, pyrazinamide and cycloserine 10° C. Relation between time and color developm Pyrazinamide is read at 490 mμ, and cycloser at 625 mμ.

PZ = 1 mM solution of pyrazinamide.
CYK = 1 mM solution of cycloserine.

the tungstic acid reagent. Under standardized conditions the loss of cycloserine can be kept approximately constant; consequently this error can be avoided by calculating the cycloserine values from a standard graph based on analyses of standard solutions of cycloserine dissolved in blood serum. Of other deproteinisation reagents perchloric acid gives a better recovery of cycloserine[2]. Pyrazinamide, however, is very unstabile in perchloric acid solution. Perchloric acid therefore cannot be used for the simultaneous determination of cycloserine and pyrazinamide.

[2] Dr. B. Sandberg A.B Kabi, Stockholm personal communication.

Specificity:

The normal serum constituents do interfere in the quantities normally pres in blood. Only sera from patients with tremely high values of non-protein nitro give blank values of 0.02—0.03 (1 cm c

A number of drugs have been exami also in respect of their possible interfere with the cycloserine and pyrazinamide de minations. It has been found that sev substances, especially cyclic compounds taining a nitrogen atom in the ring and amino group in a side chain, yield colo reaction products with sodium nitritope cyanoferroate (Fig. 4). The absorption m ima and approximate absorbance value 1 mM solutions of these substances

Table I. *Recovery experiments.*

Added mg/100 ml serum		Recovered mg/100 ml serum Cycloserine	Pyrazinamide	% recovery Cycloserine	Pyrazinamide
Cycloserine	10.0	7.6		76	
»	10.0	7.5		75	
»	10.0	7.3		73	
»	10.0	8.6		86	
»	10.0	8.1		81	
»	10.0	7.8		78	
»	5.0	3.6		72	
»	5.0	3.8		76	
»	2.5	1.8		72	
»	2.5	2.0		80	
Pyrazinamide	10.0		10.2		102
»	5.0		4.7		94
»	2.5		2.7		108
Cycloserine 10.0 + Pyrazinamide 10.0		7.6	9.8	76	98
Cycloserine 10.0 + Pyrazinamide 5.0		7.3	4.9	73	98
Cycloserine 10.0 + Pyrazinamide 2.5		7.3	2.5	73	100
Cycloserine 5.0 + Pyrazinamide 10.0		3.8	9.7	76	97
Cycloserine 5.0 + Pyrazinamide 5.0		3.7	4.8	74	96
Cycloserine 5.0 + Pyrazinamide 2.5		3.5	2.4	70	96
Cycloserine 2.5 + Pyrazinamide 10.0		2.1	9.8	84	98
Cycloserine 2.5 + Pyrazinamide 5.0		2.0	4.8	80	96
Cycloserine 2.5 + Pyrazinamide 2.5		2.0	2.5	80	100
			Mean	76.5	98.5

yn in Table II. Nicotinic acid and iso-
inic acid derivatives give absorption
ima situated so near that of pyrazin-
le that they interfere with the pyrazin-
le determination. Thiacetazone has an
rption maximum near that of cycloserine
accordingly, the latter drug cannot be
rmined in the presence of thiacetazone.
ther tuberculostatics tested, streptomy-
nd kanamycin do not cause any inter-
ce at all, and p-aminosalicylic acid only
ry high concentration.

Further investigations on the reactions between the sodium nitritopentacyanoferroate reagent and isonicotinic acid derivatives have shown that the drugs isoniazid, iproniazid and salizid can be quantitatively determined in aqueous solution by this reagent. However, it cannot be used for quantitative determination of these drugs in serum without previous extraction procedures, since, to a very large extent, they are co-precipitated with the serum proteins by the tungstic acid reagent.

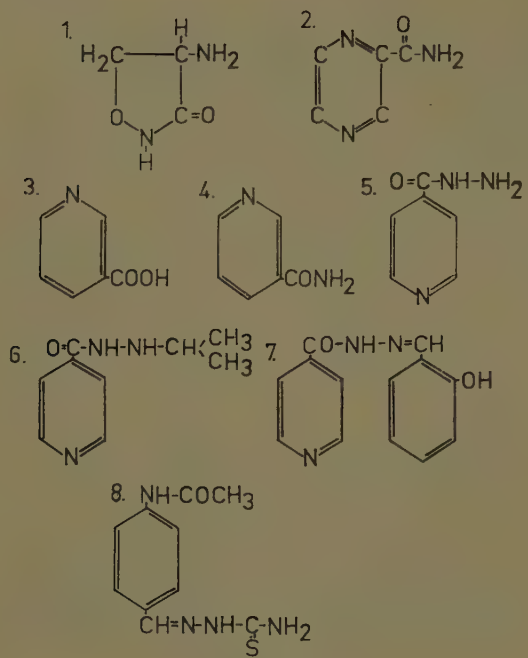


Fig. 4.

Cyclic compounds giving colored reaction products with the sodium nitritopentacyanoferroate reagent.

1. Cycloserine (D-4-amino-3-isoxazolidone)
2. Pyrazinamide (Pyrazinecarboxamide)
3. Nicotinic acid
4. Nicotinamide
5. Isoniazid (Isonicotinic acid hydrazide)
6. Iproniazid (Isonicotinic acid 2-isopropyl hydrazide)
7. Salizid (1-isonicotinoyl-2-salicylidenehydrazine)
8. Thiacetazone (p-acetamidobenzaldehyde thiosemicarbazone)

SUMMARY

Jones' original method for cycloserine analysis has been modified for the determination of pyrazinamide. In this modification the method can be used for simultaneous determination of the tuberculostatic drugs cycloserine and pyrazinamide in blood serum. Pyrazinamide can, however, not be determined in the presence of isonicotinic acid derivatives, nor cycloserine in the presence of thiacetazone.

REFERENCES

Jones, L. R.: Colorimetric determination of cycloserine, a new antibiotic. Anal. Chem. *28*, 1956.

Dahlberg, G.: Statistical methods for medical students. London 1948.

Table II. *Absorption maxima and approximate absorbance values (1 cm cell) of 1 mm standard solutions of various drugs analyzed with the sodium nitritopentacyanoferroate reagent.*

Substance	Absorption maximum mμ	Approximate absorbance value of 1 mM solutions
1. Cycloserine	625	0.60
2. Pyrazinamide.....	490	0.85
Nicotinic acid derivatives:		
3. Nicotinic acid	385	0.45
4. Nicotinamide.....	385	0.55
5. Isoniazid	440	0.80
6. Iproniazid	440	0.80
7. Salizid (Saliniazid) ..	440	0.80
8. Thiacetazone.....	635	0.60

dinav. J. Clin. & Lab. Investigation 337—340, 13, 1961.

PHENACETIN CONSUMPTION IN RELATION TO 24-HOUR ENDOGENOUS CREATININE CLEARANCE

By A. W. S. SØRENSEN

From the Danish Red Cross Rehabilitation Center, Hald, Denmark.

(Received for publication November 28, 1960)

he purpose of this paper is an evaluation idney function in patients taking phena-n.

ince Spühler (1953) described an inter-al nephritis and the possible connection this disease with phenacetin intake, a es of articles has appeared on this subject oeschlin, 1957, Larsen & Møller, 1958, deneg, Fischer, Pedersen & Nissen, 1959 others). A relationship between the de-e of anemia and the duration of phena-n intake, or between renal insufficiency the duration of phenacetin intake has er been proved (Marti, 1958). The patho-esis of the supposed kidney changes is known. Phenacetin seems to have no ect toxic effect on the kidney tissue, and ody has ever, in animal experiments, ceeded in producing kidney changes with nacetin (Gsell, 1958 and Studer, Zbin-, Schärer & Fust, 1958). With some mals Miescher, Schneider & Krech 58), however, demonstrated kidney le-ns by simultaneous administration of nacetin and injection with *bacillus coli.*

MATERIAL AND METHODS

n examinations.

he material consists of 780 patients, 265 with matoid arthritis, and 515 with other diseases 4 women and 324 men). Ages range from 17—83 years, both in the rheumatoid arthritis group, and in the non-rheumatoid arthritis group. The non-rheumatoid arthritis group will in the following be called the control group. In the control group no patients have had kidney disease, and in the rheumatoid arthritis group no kidney disease has been diagnosed before the beginning of the rheumatoid disease. The patients have all been examined by the author of this paper and questioned on their consumption of medicine containing phenacetin. All patients with a phenacetin consumption have used phenacetin until the day of examination.

Methods.

Creatinine clearance (Bonsnes & Taussky, 1945) has been carried out on all 780 patients as 3-day clearance. If a 24-hour clearance deviated more than 20 per cent from the average value of the 3-day clearance, a new 3-day clearance was carried out (Effersøe, 1956). Clearance is corrected for body surface and is given as mean value of 3-day clearance. The rheumatoid arthritis group is divided into stages of the disease according to Steinbrocker, Traeger & Batterman (1949) and this group, as well as the control group, are divided into age groups. The material is statistically treated with Student's t-test, and $p < 0.05$ is required for significant difference between two groups.

RESULTS

I. *Comparison between creatinine clearance and phenacetin intake in the control group.*

Table I shows a comparison between

Table I. *Comparison of creatinine clearance and consumption of phenacetin in the control group.*

Age group (years)	Clearance *			p	
	No consumption	Consumption below 1 gram/day up to 25 years duration	Consumption above 1 gram/day up to 30 years duration	No consumption/ consumption below 1 gram/day	No consumption/ consumption above 1 gram/day
17—40	98 ± 22 (37)	96 ± 17 (43)	97 ± 19 (26)	$p > 0.1$	$p > 0.1$
41—50	89 ± 16 (34)	91 ± 18 (53)	90 ± 14 (39)	$p > 0.1$	$p > 0.1$
51—60	86 ± 13 (51)	81 ± 12 (60)	86 ± 14 (42)	$0.05 > p > 0.025$	$p > 0.1$
>60	77 ± 13 (48)	77 ± 17 (61)	81 ± 15 (21)	$p > 0.1$	$p > 0.1$

*) Mean creatinine clearance in ml/min/1.73 m^2 ± standard deviation (No. of patients/group).

creatinine clearance and the phenacetin intake. Within each age group there is no statistically significant difference in the average kidney function between non-users and consumers with a moderate (under 1 gram per day) or large consumption (over 1 gram per day). Only in the age group 51—60 years have patients with a consumption of under 1 gram per day a significantly lower clearance than non-users, whereas no difference is found between non-users and patients with a consumption of more than 1 gram per day.

II. *Comparison between creatinine clearance and phenacetin intake in the rheumatoid arthritis group.*

Table II shows a comparison between creatinine clearance and phenacetin intake. For the age groups 17—50 and 51—60 years there is a significantly lower clearance value in patients with a consumption of more than 1 gram per day, than in non-users. There is however, no significant difference between the kidney function of non-users and users in the age group over 60 years. Table II shows a comparison between creatinine clea

Table II. *Comparison of creatinine clearance and consumption of phenacetin in the rheumatoid arthritis group.*

Age group (years)	Clearance *			p	
	No consumption	Consumption below 1 gram/day up to 28 years duration	Consumption above 1 gram/day up to 35 years duration	No consumption/ consumption below 1 gram/day	No consumption/ consumption above 1 gram/day
17—50	98 ± 24 (44)	86 ± 22 (26)	88 ± 28 (28)	$0.025 > p > 0.01$	$0.05 > p > 0.025$
51—60	82 ± 19 (36)	77 ± 19 (26)	71 ± 21 (16)	$p > 0.1$	$0.05 > p > 0.025$
>60	65 ± 22 (29)	66 ± 18 (38)	71 ± 24 (22)	$p > 0.1$	$0.1 > p > 0.05$

*) Mean creatinine clearance in ml/min/1.73 m^2 ± standard deviation (No. of patients/group).

ıce and the progressive stages of the ease. For all age groups there is a signi- antly lower clearance value in stages III + than in stages I + II. Table IV shows comparison between creatinine clearance d phenacetin intake in relation to the stage ouping in age groups under and over 50 ars. Only in the age group under 50 years there for stages III + IV, and in the age oup over 50 years for stages I + II, signi- antly lower clearance value in patients th phenacetin consumption under 1 gram r day than in non-users, whereas there is difference between patients with a con- nption of over 1 gram per day and non- ers. For stages III + IV in the age group er 50 years the kidney function of non- ers is significantly lower than that of tients with a phenacetin consumption der 1 gram per day. For the other groups significant differences between clearance lues and phenacetin consumption are found relation to the stage grouping.

DISCUSSION AND CONCLUSION

Creatinine clearance is chosen for meas- ing kidney function, as it is practical for large groups of patients (Steinitz & Türkand, 1940). In judging the possible kidney-damaging effect of phenacetin, it must be considered whether the patient's disease causes reduction of kidney function. This is true of rheumatoid arthritis as shown in Table III. In the control group phenacetin causes no reduction of kidney function (Table I). If this is compared with the rheumatoid arthritis group, where patients with large phenacetin consumption only in some groups have significantly lower kidney function than non-users, it seems that the phenacetin does not cause reduction of the kidney function, because the reduction of the kidney function caused by the disease is greater than the reduction seemingly caused by the phenacetin (Tables II and III). If the progressive stage and the phenacetin intake are considered, as shown in Table IV, only the progressive stage has any influence on the reduction of kidney function in patients with rheumatoid arthritis. Nor does the phenacetin seem to aggravate kidney function that is already reduced, because it would be expected that the average kidney function of patients in the rheumatiod arth-

Table III. *Comparison of creatinine clearance and the stages of progression in patients with rheumatoid arthritis.*

Age group (years)	Clearance *		p
	Stages I + II	Stages III + IV	Stages I + II/ stages III + IV
17—50	97 ± 21 (67)	82 ± 29 (31)	$p < 0.005$
51—60	85 ± 16 (52)	65 ± 19 (27)	$p < 0.005$
>60	74 ± 18 (37)	58 ± 25 (52)	$p < 0.005$

*) Mean creatinine clearance in ml/min/1.73 m^2 ± standard deviation (No. of patients/group).

ritis group with large phenacetin consumption of over 1 gram per day for many years would be significantly lower than in non-users within each stage, but this is not the case (Table IV).

SUMMARY

The object of this paper is an evaluation of kidney function in patients with phenacetin intake. The examination covers 780 patients, 265 with rheumatoid arthritis, and a control group of 515 patients with other diseases, on whom 3-day endogenous creatinine clearance has been carried out.

Neither in the control group nor in the rheumatoid arthritis group is reduction of kidney function caused by phenacetin. Nor does phenacetin seem to aggravate an already reduced kidney function.

REFERENCES

Bonsnes, R. W. & Taussky, H. H.: On the colorimetric determination of creatinine by the Jaffe reaction. J. Biol. Chem. *158,* 581, 1945.

Effersøe, P.: Relationship between endogenous 24 hours creatinine clearance and serum creatinine concentration in patients with chronic renal disease. Acta med. Scandinav. *156,* 429, 1956.

Gsell, O.: Primär chronisch interstitielle Nephritis in Beziehung zum Phenacetinabusus. Bul schweiz. Akad. d. med. Wissensch. *14,* 12 1958.

Larsen, K. & Møller, C. E.: Nyrelidelse foraa saget af phenacetinmisbrug. Ugesk. f. læge *120,* 114, 1958.

Lindeneg, O., Fischer, S., Pedersen, J. & Nisse N. I.: Nyrepapilnekrose og langvarig phenac tinmisbrug. Ugesk. f. læger *121,* 532, 1959.

Marti, H. R.: Die Anämie bei chronischem Phe acetinabusus. Schweiz. med. Wchnschr. *8* 1054, 1958.

Miescher, P., Schnyder, W. & Krech, U.: Z Pathogenese der "interstitiellen Nephritis" b Abusus phenacetinhaltiger Analgetica. Schwei med. Wchnschr. *88,* 432, 1958.

Moeschlin, S.: Phenacetinsucht und -schäde Schweiz. med. Wchnschr. *87,* 123, 1957.

Spühler, O.: Probleme der interstitiellen Nephrit Schweiz. med. Wchnschr. *83,* 145, 1953.

Steinbrocker, O., Traeger, C. H. & Batterma R. C.: Therapeutic criteria in rheumatoi arthritis. J.A.M.A. *140,* 659, 1949.

Steinitz, K. & Türkand, H.: The determination the glomerular filtration by the endogeno creatinine clearance. J. Clin. Investigation 285, 1940.

Studer, A., Zbinden, G., Schärer, K. & Fust, E Tierexperimentelle Untersuchungen zur Fra der interstitiellen Nephritis bei Missbrau Phenacetinhaltiger Schmerzmittel. Bull. schwe Akad d. med. Wissensch. *14,* 659, 1958.

Table IV. *Comparison of creatinine clearance and consumption of phenacetin in relationship to stages of progression in the rheumatoid arthritis group.*

Stages	Age group (years)	Clearance* No consumption	Clearance* Consumption below 1 gram/day up to 28 years duration	Clearance* Consumption above 1 gram/day up to 35 years duration	p No consumption/ consumption below 1 gram per day	p No consumptio consumption above 1 gram per day
I + II	< 50	101 ± 24 (31)	92 ± 18 (19)	95 ± 18 (17)	0.1 > p > 0.05	p > 0.1
I + II	> 50	83 ± 16 (42)	76 ± 19 (29)	84 ± 21 (18)	0.05 > p > 0.025	p > 0.1
III + IV	< 50	92 ± 22 (13)	70 ± 23 (7)	79 ± 45 (11)	0.05 > p > 0.025	p > 0.1
III + IV	> 50	53 ± 30 (22)	67 ± 18 (36)	62 ± 12 (20)	0.025 > p > 0.001	p > 0.1

*) Mean creatinine clearance in ml/min/1.73 m^2 ± standard deviation (No. of patients/group).

ındinav. J. Clin. & Lab. Investigation 341—345, 13, 1961.

HISTORY OF THE INTERNATIONAL FEDERATION OF CLINICAL CHEMISTRY

By E. J. KING,

From the Postgraduate Medical School, London, W. 12, England

(Received for publication November 14, 1960)

In September 1951 the International Union of re and Applied Chemistry (IUPAC) held a Conence and Congress in New York. Professor Tiselius, then President of the Biological Chemry Section of IUPAC became President of the ion, and Sir Charles Dodds (then Professor C. Dodds) succeeded him as head of the Biolo- al Chemistry Section, with Professor Murray ck as Secretary. The writer was in New York this time, at the invitation of the American sociation of Clinical Chemists, and was apached by these representatives of the Biological emistry Section of IUPAC to join and take on Presidency of the Commission of Clinical Chemry, to consist of himself, Professor Warren Sperry .S.) and Professor Paul Fleury (France). Tise-, Dodds and Luck felt that there was a need for international body, with the backing and support a Union, to pull together and correlate the actiies of the very few known national societies of nical Chemistry, and to encourage the formation such societies in other parts of the world; furrmore, that the chemists and biochemists who rked in hospitals and the clinical departments of dical schools were now sufficiently numerous, at st in the principal countries, to warrant drawing m together into some sort of loose organization l to periodic meetings which might well be on international scale.

During the following winter of 1951/52 correspondence took place between King, Sperry and Fleury as to possible avenues of profitable activity. An invitation was then received from the Netherlands Society of Clinical Chemistry that an international congress be held in Amsterdam in the spring of 1952, to which the clinical chemists of the world would be invited, and which should be the occasion for the foundation of an international organization of clinical chemists, as well as forming a congress for the presentation of lectures and research communications. Negotiations were already well forward with the French clinical chemists and biochemists to organize and strongly back a medical and clinical section of the International Congress of Biochemistry planned for Paris in August of that year. It was represented to the Dutch chemists that the International Congress in Paris would be attended by biochemists and clinical chemists from all over the world, and that this would probably be a more favourable occasion and place to launch a new International Federation of Clinical Chemistry than would Amsterdam in the the spring. It is to the very great credit of the Netherlands Society that they immediately agreed to withdraw their proposal and to meet with the other clinical chemists of the world in Paris that summer.

The members of the IUPAC Commission on Clinical Chemistry (Fleury, King and Sperry),

with the informal support of representatives of other known existing national societies, organized a meeting for the afternoon of July 24th in the Sorbonne. There was a large attendance by clinical chemists from many countries, several of them the official spokesmen of national societies. Dr. I. D. P. Wootton (London) acted as Secretary, and Dr. Ramon of Fleury's Department, an accomplished linguist, acted as interpreter. There was enthusiastic support for the proposal to found an International Federation of Clinical Chemistry, and to set up a Committee of persons who should be named as the official representatives of existing national societies. It was further agreed that the Commission of Clinical Chemistry of IUPAC should be enlarged to include these national representatives, and that it should act as the Committee of the new International Federation of Clinical Chemistry. No constitution or settled rules were envisaged for the new Federation at that time, but a set of aims and objects was discussed and the following unanimously agreed:

1. An International Association of Clinical Biochemists shall now be formed, whose function shall be to advance knowledge in and promote the interests of biochemistry in its clinical (medical) aspects.

2. The Commission on Clinical Chemistry of the Section of Biological Chemistry of the International Union of Pure and Applied Chemistry shall act initially as the Committee of this International Association of Clinical Biochemists. The Committee shall subsequently include representatives from national societies.

3. The Committee shall set up Sub-Committees to deal with specific matters of a special nature (e. g. with internationally agreed ways of expressing results), as may seem indicated from time to time.

4. The Committee shall make attempts to ascertain the titles of existing Societies of Clinical Biochemistry (or Societies serving the same function under different names) and of their officers, and it shall endeavour to promote the foundation of societies in countries where they do not already exist.

5. From the lists of names of members of existing societies and from the names of such other persons as may be ascertained to be practising clinical biochemistry, the Committee shall prepare an international list of Clinical Biochemists, with their addresses and where ascertainable, their principal scientific interests.

6. The International Association of Clinical Biochemists will meet during and before and after if deemed advisable the International Congresses of Biochemistry and its members will use the Clinical Chemistry section of the Congress as a forum in which to present their communications.

7. The Committee shall represent to the local organizing committee of the Congress that symposia on subjects of special interest to clinical biochemists be held during the Congress and possibly during a day (or two) preceding the Congress.

8. The Committee shall encourage and attempt to promote regional meetings on a semi-international scale.

9. The Committee shall receive and circulate information regarding special new techniques and other matters of interest which might not be readily available through the ordinary vehicles of publication.

10. The Committee or a Sub-Committee shall arrange for the circulation of solutions and samples for the comparison of methods and results and shall attempt to standardize the expression of results of such methods as are expressed in units or other arbitrary terms.

In the summer of 1953 a new enlarged Commission and Committee met in Stockholm, at the International Congress of Chemistry which was being held there. The following attended and were received into full Titular Membership of the Commission on Clinical Chemistry by the Biological Section of IUPAC.

E. J. King, President (U.K.)
I. D. P. Wootton, Secretary (U.K., rep. Brit. Assoc. Clin. Biochem.)
P. Fleury (France, rep. Soc. Fr. Biol. Chim.)
A. Sobel (U.S.A., rep. Amer. Assoc. Clin. Chem.)
B. Josephson (Sweden, rep. Scand. Soc. Clin. Chem. & Clin. Physiol.)
J. C. M. Verschure (Netherlands, rep. Neth. Soc. Clin. Chem.).
W. M. Sperry (U.S.A.) was unable to attend.

At their meetings the new Commission and Committee agreed to attempt the organization of a regu- congresses of Clinical Chemistry at two or three ar intervals, to encourage the holding of regional mi-international meetings of clinical chemists in jacent countries, to promote the foundation of new tional societies of Clinical Chemistry in countries here there appeared to be sufficient numbers of terested persons and of "groups" of clinical emists within other societies, biochemical, bio- gical, chemical, pathological, etc., where the mbers were not sufficient to make viable inde- ndent societies, to set up an international haemo- obin standard in co-operation with the Protein ommission of IUPAC, and to conduct an inter- tional trial of analytical methods commonly used the clinical laboratory to compare the results tained in different laboratories of the countries presented.

The first International Congress of Clinical hemistry was appropriately held in Amsterdam in ptember 1954. It was an outstanding success. eople attended from almost every country in the orld. There were over five hundred registered rticipants, and while most of them were from estern Europe, there were several from North merica, and a few from as far away as Australia, India and South America. The organized mposia set a high scientific standard, and the ort communications offered were of wide interest. r. J. C. M. Verschure and Dr. Th. Strengers President and Secretary) and their Committee oduced a model of organization and successful nduct which succeeding congresses have not ex- lled.

At this meeting an informally convened group National Representatives considered a proposal om the publishing firm of Elsevier that the newly- unded Journal "Clinica Chimica Acta" should be- me the official organ of the International Fede- tion of Clinical Chemists. While there was some pport for the idea in Western Europe, it was felt those countries which already had their own urnals (e. g. Scandinavia, U.S.A., etc.) that it ould not be wise for the new International Fede- tion to have a new single publication of its own; t that the Federation should give its full moral pport and backing to Clinica Chimica Acta.

For the Biochemical Congress in Brussels in 1955, a strong Clinical Chemistry section was organized, Committee meetings of the Commission and Committee was held, and there was a General Meeting of the Federation, which was well attended, like those in Amsterdam and in Paris. There was also a very successful banquet, organized by the Belgian Clinical Chemists, at which over a hundred attended.

Colonel M. E. Freeman replaced Dr. A. Sobel as the American representative in 1955.

An American invitation for a congress in New York in 1956 had been received in Amsterdam in 1954 and its acceptance was confirmed in Brussels in 1955.

The Second International Congress of Clinical Chemistry was held under the auspices of the American Association of Clinical Chemists in the Park Plaza Hotel in New York in September 1956. Dr. Albert Sobel was the President, Dr. Max Friedman (Secretary A.A.C.C.) and the American Committee furnished an excellent programme in symposia and several sections, and the excellent entertainment culminated in a banquet.

A Scandinavian invitation to a congress in Stockholm in 1957 was received and accepted at this time. It met under the Presidency of Professor J. Lehmann, and this Third International Congress was as successful as its two predecessors.

In 1958 the Austrian organizers of the Biochemical Congress in Vienna arranged for a Clinical Chemistry section to be held within the Congress and for a banquet for the Federation. A General Meeting was held after one of the scientific sessions, when the Committee again reported on the activities of the Federation, as it had done at previous Congresses. Several changes were made in the composition of the Commission and Committee: Dr. Wootton resigned as Secretary and was replaced by Colonel M. E. Freeman (U.S.A.); Professor N. F. Maclagan (U.K.) succeeded Dr. Wootton as the representative of the British Association of Clinical Biochemists; Professor J. Courtois succeeded Professor Paul Fleury as representative of the Société Française de Biologie Clinique; Professor K. Hinsberg became the representative of Germany; and Professor V. Orekhovich representative of the U.S.S.R. Dr. S. H. Jackson

had been suggested from Canada as the representative of their Society of Clinical Chemistry, Professor R. Ruyssen from Belgium, and Dr. M. C. Sanz from Switzerland.

At the IUPAC Conference held in Munich in August 1959, these names were submitted to and agreed by the Biological Section and the Council of IUPAC. Prof. Sperry retired under the IUPAC eight year rule. Prof. King resigned and Colonel Freeman succeeded him as President. The full composition of the Commission, subsequently confirmed as the Committee at the general meeting in Edinburgh in 1960, was therefore as follows:

Titular Members: Freeman, M. E., U.S.A., President, Josephson, B., Scandinavia, Secretary, Courtois, J., France, Hinsberg, K., Germany, Jackson, S. H., Canada, Maclagan, N. E., U. K., Orekhovich, V., U.S.S.R., Verschure, J. C. M., Netherlands.

National Representatives: Ruyssen, R., Belgium, Sanz, M. C., Switzerland.

Note: Titular members of a IUPAC commission receive their travelling expenses to meetings; their number is limited to eight. National representatives are full members of the commission, but do not receive their travelling expenses.

For 1960 an invitation had been sent from the British Association of Clinical Biochemists to hold a congress of Clinical Chemistry in Edinburgh. This was received at the meeting in New York in 1956, and was confirmed in Vienna in 1958. Under the Presidency of Sir Rudolph Peters a highly successful Congress of symposia, communications and demonstrations was held in the week 14th—19th August, 1960. There were nearly 600 registered participants. Dr. C. P. Stewart (Chairman), Dr. S. C. Frazer (Secretary Organizing Com.), and Dr. B. C. Frazer lyn (Sec. Prog. Com.) provided excellent entertainment as well as an outstanding scientific programme. At the Commission and Committee meetings it was reported that the Sub-Commission on Clinical Enzymes had met at Munich and had recommended that enzyme activities be reported in terms of micro moles of substrate transformed per min. per litre; that a new scheme was in hand to despatch accurately analysed samples of freeze-dried blood plasma to selected laboratories in different countries to establish uniformity of analytical results; that an invitation had been received from the Canadian Society of Clinical Chemistry and the American Association of Clinical Chemists to hold a Congress in a border city in 1963; that in addition to the countries already having National societies of clinical chemistry, Yugoslavia, Portugal, Japan and Australia were forming new societies; agreed that a sixth congress be held in Southern Europe in 1966; that a Directory of member societies, including names and addresses of officers, be prepared; and that the officers of the Commission and Committee should now draw up a constitution and bye-laws for the International Federation of Clinical Chemistry, which should be sent to the adhering national societies and subsequently discussed at ensuing Commission and Committee meetings and at the next General Meeting to be held, probably in a U.S.A.-Canadian border city. The following motion was put to the General Meeting by the Commission and Committee and was agreed: be it resolved that the retiring Committee of the International Federation of Clinical Chemistry (IFCC) does now recommend to the IFCC the the arrangement begun in Paris 1952, and subsequently confirmed in Amsterdam in 1953, Brussels 1955, New York 1956, Stockholm 1957, and Vienna 1958, whereby the Commission on Clinical Chemistry of IUPAC acts as the Committee of the IFCC be confirmed and continued.

It is further recommended that in the future as in the past, the membership of the Commission and Committee be reviewed at each of its meetings in the light of the wishes of the National Societies of Clinical Chemistry, in order that proper representation of International Clinical Chemistry may be maintained, and that changes recommended shall be presented to the next ensuing General Meeting of the IFCC and to the Biological Section of IUPAC for confirmation.

SUMMARY

A Commission on Clinical Chemistry was formed in the Biological Section of IUPAC in 195 The International Federation of Clinical Chemist (IFCC) was founded in Paris in 1952. Internation Congresses of Clinical Chemistry have been he in Amsterdam (1954), New York (1956), Stoc

n (1957), and in Edinburgh (1960). Sections of ical Chemistry have been organised (with the peration of the Committee of IFCC) in the rnational Biochemical Congresses in Paris 2), Brussels (1955), and Vienna (1958). The mission on Clinical Chemistry acts as the Com- ee of the IFCC, and holds its meetings both he International Congresses of Clinical Chem- y and (usually) during the International Con- sses of Biochemistry. The activities of the IFCC include the holding of congresses, the setting up of standards of reference, e. g. haemoglobin, international trials of methods of analysis in clinical chemistry, the organization of schemes for the preparation of standard sera for analyses, the encouragement of scientific activities with national societies, the formation of new societies intercourse between them, and the holding of symposia on subjects in clinical chemistry organized by societies within countries as well as internationally.

Scandinav. J. Clin. & Lab. Investigation 346, 13, 1961.

CLINICAL ENZYME UNITAGE

By M. E. FREEMAN

From the Sub-Commissions (of IUPAC and IUB) on Clinical Enzyme Units, River House A 202, 1400 South Street, Arlington, Virginia, U.S.A.

Interpretation and translation of many arbitrary, varied and unrelated clinical enzyme units in current use has been a source of misunderstanding and confusion among clinical chemists. This will continue to be a vexing problem until steps are taken to reach some agreement on a common unitage. The problem is obviously complicated by crude reactants and poorly defined reactions. It is, therefore, manifestly impossible to reach a completely satisfactory solution in every case. However, it was the considered opinion of the Joint Sub-Commission on Clinical Enzyme Units of the International Union of Biochemistry and the International Union of Pure and Applied Chemistry, meeting in Munich in 1959, that some clarification should be attempted, that dimensions should be in basic units, clearly defined and acceptable to all chemists and clinicians. The conclusion and recommendation of the Joint Sub-Commission was that, wherever practicable, clinical enzyme units should be difined as micro-moles of substrate transformed per minute under specified conditions, and their concentration in terms of a millilitre (or litre) of serum, plasma or urine. Where thi is impossible or difficult because of the com plex nature of the substrate and uncertaint as to the nature of the products, e. g. wit fats, proteins or starch, the units should b expressed in terms of the analysable sub stances or groups determined to measure th reaction e. g. fatty acid, amino or carboxy groups and reducing groups. Thus, for in stance, with a phosphatase the enzym activity can be expressed as micro-moles o organic phosphate hydrolysed (i. e. of phos phate liberated per minute per millimetr (or litre); with amylase as micro-moles o reducing group.

This recommendation is in conformanc with the report of the Enzymes Commissio of the International Union of Biochemistry

REFERENCES

Report of the International Commission of I.U.B on Enzymes, Pergamon Press, to be publishe 1961.

Hoffman-Ostenhoff, O. & Thompson R. H. S Nature *181*, 452, 1958.

Technique and Practical Problems

ndinav. J. Clin. & Lab. Investigation 347—348, 13, 1961.

A SIMPLE MECHANICAL STIRRING DEVICE

ITS APPLICATION TO RAPID DETERMINATION OF CARBONATES BY A MODIFIED CONWAY TECHNIQUE

By I. HOLM-JENSEN

From the Pharmacological Institute, University of Aarhus, Denmark.

(Received for publication October 6, 1960)

Analytical procedures selected for use in nical routine should require a minimum of our and time expended on each analysis, d, preferably, the results should be ained within a short time.

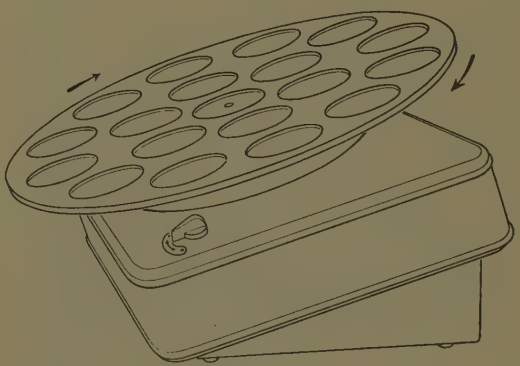
The application of the Conway micro-fusion technique for the determination of bonates and ammonia (urea) in biological ids is, in practice, hampered by the fact t the time required for these analyses as formed in most laboratories is between 60 d 90 minutes.

Conway (1957) points out that an in-ased rate of absorption can be obtained rocking or vibrating the Conway units ring the diffusion period. However, he ommends the use of stirring arrangements y with some reservation: "In general, king is not advised except for special pur-ses, since it will obviously tend to diminish working ease and simplicity of the thod; where, however, suitable shaking vices are at hand their use may quite often ve helpful."

A mechanical stirring device which is table for use in several of the Conway cro-diffusion analyses is described below. application offers the advantages that manual stirring for mixing samples and reagents becomes superfluous, and that the diffusion time is often greatly reduced.

The stirring arrangement consists of a circular disc provided with recesses to accommodate the Conway units. The disc is placed at an angle of 18° to the horizontal plane. The stirring is accomplished by rotating the disc around its axis at a rate of about 16 revolutions per minute.

On the basis of experiments, the slope of the disc and the rate of rotation given above



The proposed turntable stirring device. For explanation, see text.

were selected as a safe compromise with regard to stirring efficiency and risk of accidental exchange of fluid between the outer and inner chambers of the Conway units. The maximum volumes of fluid in the inner chambers of the Conway units Nos. 1 and 2 should not exceed 1.0 ml and 0.5 ml, respectively.

The stirring arrangement (Fig. 1) is made from a 4-speed gramophone from which the tone arm is removed. The speed regulator is permanently adjusted to 16 revolutions per minute. The gramophone iis fixed to a wooden support in such a way that the turntable forms an angle of 18° to the horizontal plane.

The disc for accommodating the Conway units is made of two layers of "Perspex" cemented together. The upper layer is furnished with 19 evenly spaced holes with diameters of 66.5 mm, corresponding to the Conway units No. 1. The disc has a central bore for the protruding shaft of the gramophone turntable. For accommodating the smaller Conway units, No. 2, rings of "Perspex" with inner diameters of 42.5 mm, corresponding to the outer diameters of these units, are placed in the recesses of the disc.

The above stirring device has been used in different Conway micro-diffusion analyses. Its application in the modification of the original Conway procedure for the determination of carbonates in 0.1 ml samples of blood plasma recently described by the writer (Holm-Jensen 1960) is briefly discussed below.

The first analyses were carried out on 0.1 ml samples of a 25 mM $NaHCO_3$ solution. The analyses were performed as described in the aforementioned paper with 0.5 ml of 10 mM $Ba(OH)_2$ as absorbent, but without manual stirring. The units were placed on the rotating disc of the stirring apparatus during diffusion periods of different lengths. In diffusion periods of 10, 30, 60, and 180 minutes, the quantities of CO recovered were about 85, 99, 100, and 10 per cent, respectively.

In subsequent experiments, the transfe of CO_2 was studied under varying experi mental conditions. It was found that by re placing the 0.5 ml 10 mM $Ba(OH)_2$ solu tion so far used for the absorption of CO by 0.4 ml of a 25 mM solution of the hydrox ide the 10-minute transfer increased to abou 96 per cent, and with this absorbent th transfer in 15 minutes was determined a 98.8 $\pm$ 0.8 per cent; in 20 minutes it wa 99.6 $\pm$ 0.8 per cent.

From the above findings it seems reason able to use 0.4 ml 25 mM solution o $Ba(OH)_2$ as absorbent and a diffusion perio of 15 minutes (corresponding to the transfe of 99 per cent of the CO_2) for routin analyses.

The titrations may be carried out b means of the 0.1 ml burette with 0.2 standard hydrochloric acid. The bicarbona concentration of the sample expressed i milliequivalents per litre will then be equ to the difference, in µl, between the quan tities of standard acid used for titration the blind and the sample analysis.

The procedure proposed above was finall applied to the determination of bicarbona in human blood plasma; it was found th also in this analysis a diffusion time of 1 minutes was sufficient for the transfer of 9 per cent of the CO_2.

The present study was aided by a grant fro King Christian X's Foundation.

REFERENCES

Conway, E. J.: Microdiffusion Analysis and Vol metric Error, 4th edition, Crosby Lockwo & Son, London 1957.

Holm-Jensen, I.: A modified Conway technique f the determination of carbonates with titrati carried out during magnetic stirring in a ca bon dioxide-free atmosphere. Scandinav. Clin. & Lab. Investigation *12*, 269, 1960.

ndinav. J. Clin. & Lab. Investigation 349—350, 13, 1961.

A GASOMETRIC APPARATUS FOR DIRECT READING DETERMINATION OF CARBON DIOXIDE CONCENTRATION IN GAS MIXTURES

By O. SIGGAARD ANDERSEN and K. JØRGENSEN

From the Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark.

(Received for publication September 28, 1960)

Determination of the blood acid-base tus by means of equilibration of blood or sma with CO_2 of known partial pressure iggaard Andersen, Engel, Jørgensen & trup 1960) has, during the last few years, ınd increasing application in routine work. ere is, consequently, an increasing need a simple method for determination of the O_2 concentration in gas mixtures. We have ınd a modified Hempel (or Orsat) appa-us practical. A description of the Hempel paratus can be found in the Textbook of ıantitative Inorganic Analysis (Vogel, 51). The principle of the analysis is a easurement of the diminution in volume ieasured at atmospheric pressure) after sorption of CO_2 in sodium hydroxide. In original method the gas mixture is ad-sted to atmospheric pressure after CO_2 sorption by levelling the surface in the elling bulb with that of the burette fluid. is levelling may be somewhat difficult and ıccurate especially when the burette has pillary dimensions. The difficulty is avoid-in the modification described here by ng the absorption fluid as manometer id.

The apparatus consists of a water-keted 10 ml pipette of which the lower l forms a calibrated burette graduated from zero to 1.5 ml. The lower end of the pipette is connected through a stopcock (B) with a levelling bulb containing 0.1 M sulphuric acid. The top of the pipette is connected through the stopcock (A) with either the CO_2 absorption system (position 2) or with the tube where the gas mixture can be admitted (position 1). The CO_2 absorption system forms a detachable U-tube (see figure) connected with the apparatus by means of a ground ball joint. The U-tube is filled with enough sodium hydroxide for the surfaces to reach the middle of the two narrow parts of the U-tube. The right side of the U-tube is filled with a bundle of glass tubes to increase the absorbing surface; the left side is surmounted by a soda-lime-filled tube.

The tube for the gas admission is provided with a small bubbling bulb filled with water to indicate the rate of the gas admission.

The level of the sulphuric acid in the pipette is regulated by means of the levelling bulb and the stopcock B. Care must be taken never to raise the sulphuric acid further than the level of stopcock A, and, likewise, never to suck the sodium hydroxide further than stopcock A.

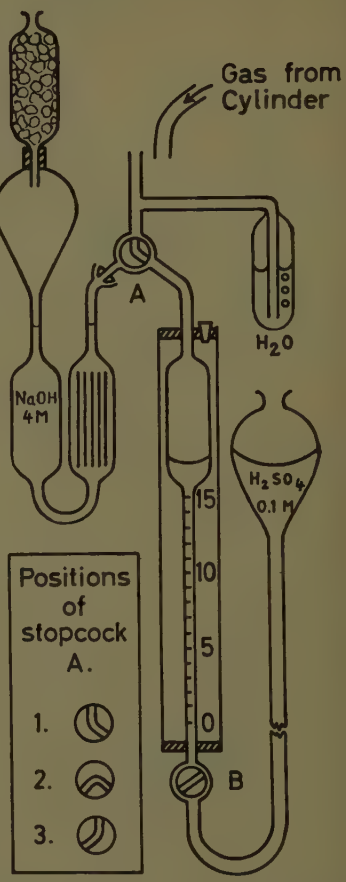
Before use, it is necessary to moisten the upper left side of the U-tube with sodium

hydroxide to insure complete return during the analysis. This is done by raising the sulphuric acid from the lower end of the pipette with the stopcock A in position 2. With stopcock A in position 3, the sodium hydroxide is next levelled with a pressure of one atmosphere above both surfaces. The surfaces do not always level exactly because of different surface tensions due to the presence of carbonate on the right side. The accurate levels of the surfaces are marked.

With stopcock A in position 1, the tube from the gas cylinder is now connected with the apparatus and the gas admitted. The pipette is washed a few times with the gas and then filled slowly to the zero point (slowly to ensure that the gas mixture is saturated with water vapor at the temperature of the pipette). Now the tube from the gas cylinder is removed so that the gas mixture attains atmospheric pressure. With stopcock A in position 2, the gas mixture is now gently pushed 5 times to and fro between the absorption tube and the pipette by means of the levelling bulb. The last time the sulphuric acid is lowered slowly until the surfaces of the sodium hydroxide in the U-tube have exactly the same position as marked at the beginning of the analysis. The pressure of the gas in the cylinder is then one atmosphere and the CO_2 concentration of the gas mixture (concentrations reaching from 0 to 15 per cent) can be read directly in volumes per cent on the burette.

One source of error is a change in temperature of the gas during the measurement. To prevent this error the apparatus must be kept away from draught and warming of the sulphuric acid in the levelling bulb by hand be avoided. Another source of error is the absorption of other gases in the gas mixture, *e. g.* oxygen, due to high partial pressures. This error will be revealed if, after the first reading, the absorption procedure is repeated and new readings taken. If these readings show a significant drift the error on the first reading may be corrected by extrapolation.

The standard deviation on a single determination calculated from 20 double analyse of different gas mixtures containing fro 2.63 per cent CO_2 to 9.51 per cent CO_2 wa 0.013 per cent CO_2.



Modified Hempel (or Orsat) apparatus.

REFERENCES

Siggaard Andersen, O., Engel, K., Jørgensen, J & Astrup, P.: A micro method for the dete mination of pH, carbon dioxide tension, ba excess and standard bicarbonate in capilla blood. Scandinav. J. Clin. & Lab. Investigatio *12*, 172, 1960.

Vogel, A. I.: Textbook of Quantitative Inorgan Anaysis. Theory and Practice. Longmans, Green & Co., London, II ed. 195 p. 784 f. f.

ndinav. J. Clin. & Lab. Investigation 351—352, 13, 1961.

RECTANGULAR CUVETTES AS SELF-EMPTYING CELLS

By K. JØRGENSEN

From the Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark

(Received for publication September 28, 1960)

n colorimetric routine work the advan-
es of self-emptying types of cells are well
ognized. In their usual forms these cells
cylindrical tubes with funnel-shaped
tom outlets and require at least 4 to 5 ml
solution for measurement.
n our laboratories the greatest part of the
rimetric analyses are performed on a

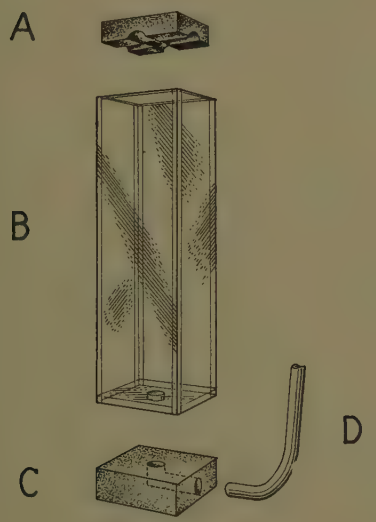


Fig. 1.

-emptying cell, exploded view. An ordinary
re cuvette, B, provided with a bottom hole,
lued on to the piece of polyvinyl-chloride, C,
which bore the polyethylene tubing, D, fits
ly. The small block, A, of polyvinylchloride
the like) is placed loosely at the bottom of B.

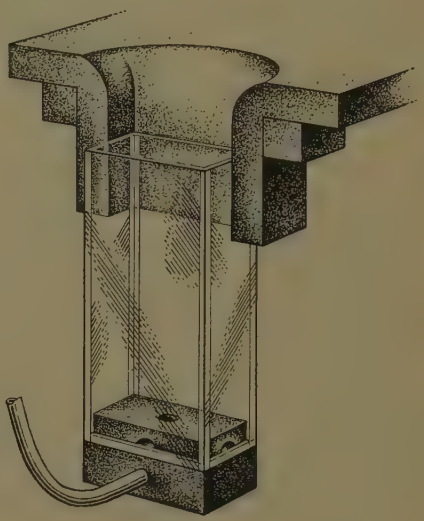


Fig. 2.

The mounting of a self-emptying cell. The cuvette (a 20 mm cell is shown) is lengthened with walls of polyvinylchloride glued on to the glass. The plastic walls continue into a cuvette housing lid (shown in vertical section).

micro scale with all operations carried out in disposable glass tubes, 80 × 10 mm. (The tubes are packed and delivered dustless from the manufacturer to be used without previous rinsing.) Accordingly, our final volumes are about 2 to 3 ml. For such volumes self-emptying cells have been made, following the principles given below. They function well, *even as 20 mm cells, e. g.* in connection with

the Beckman photometers type DU, B and C.

Rectangular cuvettes are preferred to round ones as they allow the greatest proportions of their content to be exposed to the light beam. In addition the thickness of absorbent is well defined. However, a square cell is difficult to empty by suction as the fluid tends to be retained in the corners. This difficulty is overcome as shown in Fig. 1. The capillary space around the block inserted, together with its central hole, drains the cuvette efficiently when suction is applied.

Cuvettes according to Fig. 1 can be placed in the conventional holders and the solu tion be furnished through some funnel in th lid of the cuvette housing. It is better, how ever, to make a special arrangement a shown in Fig. 2. The actual appearance c the set-up will depend on the photomete used. A diaphragm must eventually be in serted to limit the height of the light beam For very sensitive photometers the funnel shaped opening must be covered durin measurement. When polyvinylchloride i used only an insignificant amount of fluid i retained in the funnel.

Letters to the Editors

candinav. J. Clin. & Lab. Investigation 353—354, 13, 1961.

THE CONTENTS OF GLYCOGEN AND POTASSIUM IN HUMAN LIVER TISSUE OBTAINED BY NEEDLE BIOPSY

Earlier determinations of glycogen and otassium in human liver tissue have been erformed in post-mortem material or in ecimens obtained at operations. As far as e know no analyses of this kind have been erformed in needly biopsy material from ealthy subjects. However, glycogen analyses n needle biopsy material from hospitalized atients have been carried out by Hildes, herlock & Walshe 1949.

We have examined biopsy material from x healthy volunteers (4—9 in the table) nd three patients with chronic polyarthritis —3). One of these (1) had an advanced ver damage (pre-cirrhotic stage according histological examinations) the other two owed only slight unspecific inflammatory ver changes. All subjects had normal serum ectrolytes.

All biopsies were performed between 10 nd 11 a. m. The subjects had not taken any od since the previous evening. A siliconized im—Silverman needle was used for the ver biopsies. Special care was taken not to filtrate either the capsula or the liver tissue ith local anesthetic.

Wet weight of the biopsy material ranged tween 10 and 20 mg. The blood was wiped vay and visible connective tissue was rapidly removed by dissection. The material was divided into two pieces, one for water and fat determination, the other for glycogen, potassium and protein. For wet weight determination each piece was stuck to a platinum hook and repetedly weighed on a micro balance during the first five minutes. By extrapolating to zero time the original wet weight was estimated.

The piece intended for water and fat determination was dried in an oven at 90 centigrades for twenty-four hours and then reweighed. The water content was calculated. The fat was extracted with petroleum ether and the weight decrease was calculated.

The other piece was immediately homogenized in a plastic homogenizer containing 2 ml distilled water. One ml of the homogenate was used for potassium and protein determination and the rest for glycogen analysis.

For protein determination sodium hydroxide solution was added to a final concentration of 50 mEq/l. After 18 hours standing at room temperature the sample was centrifuged and the alkali soluble protein determined (Lowry, Rosebrough, Farr & Randall 1951). Potassium was determined in the same solution by use of flame photometry.

The glycogen was determined as trichlor-

acetic acid extractable hexose-polysaccharides. 30 per cent trichloracetic acid was added to the homogenate giving a final concentration of 10 per cent and the solution was centrifuged. The glucose concentration in the supernatant was determined before and after hydrochloric acid hydrolysis, the orthotoluidin method for glucose beeing employed (Hultman 1959). Glycogen solutions treated in the same way were used as standards.

The results are presented in the table. The glycogen and potassium contents were referred to wet weight and to alkali-soluble protein content. In the statistical treatment subject I was excluded because of the histological findings.

A more extensive report will be published later, in which the details of the methods will be given and the results discussed.

The investigation was supported by grants from «John Bergwalls Foundation» and the «Swedish Association Against Rheumatism».

REFERENCES

Lowry, O. H., Rosebrough, N., Farr, L. & Randall, R.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265, 1951.

Hildes, J. A., Sherlock, S. & Walshe, V.: Liver and muscle glycogen in normal subjects, in diabetes mellitus and in acute hepatitis. Part I. Clin. Sci. *7*, 287, 1949.

Hultman, E.: Rapid specific method for determination of aldosaccharides in body fluids. Nature *183*, 108, 1959.

Table I. *Results of analysis of live biopsy specimens. 1—3 chronic polyarthritis. Case 1 complicated with pre-cirrhotic liver damage. 4—9 healthy subjects.*

Subject	Sex	Age	Serum K mEq/l	g/100 g wet weight			g glycogen /g protein	K mEq/100 g wet weight	K mEq/g protein
				H_2O	protein	glycogen			
1	M	47	4.8	74.4	16.3	2.26	0.14	8.52	0.53
2	F	60	4.4	64.5	16.0	4.25	0.27	8.42	0.53
3	F	51	4.4	—	18.3	4.43	0.24	9.44	0.52
4	M	24	4.9	71.8	18.3	4.04	0.22	9.41	0.52
5	M	27	4.8	69.1	18.3	5.60	0.31	8.48	0.46
6	M	25	4.5	69.8	16.1	4.71	0.29	9.47	0.59
7	M	28	4.9	71.7	19.3	5.82	0.30	7.18	0.37
8	M	38	4.0	69.0	14.9	6.11	0.41	7.60	0.51
9	M	28	4.3	70.3	—	4.73	—	—	—
Number of determinations*				7	7	8	7	7	7
Mean value				69.5	17.3	4.96	0.29	8.57	0.50
Standard deviation				2.5	1.63	0.78	0.062	0.93	0.069

* Subject 1 excluded.

The Central Clinical Laboratory
S:t Eriks Hospital,
Stockholm, Sweden.
January 2, 1961.

J. Bergström
J. Findor
E. Hultman.

andinav. J. Clin. & Lab. Investigation 355, 13, 1961.

THE USE OF IMMERSED TEST TUBES AS PHOTOMETER CELLS

In the Stegemann (1960) version of amino id separation on resin columns, the ana- tical procedure is simplified by performing e ninhydrin color development in the same st tubes used for the collection of the 1 ml mples. The very same tubes are transferred rectly from the waterbath to the Elko-III pe of photometer in which the space around e tube has been filled by water. Drying or iping of the tubes which normally is a time- nsuming procedure, is unnecessary.

The use of water instead of air in the space tween the light source and the photo cell ay have several advantages, and it may be ed in other types of photometers as well if closely fitting water container is inserted in e apparatus.

The figure illustrates a very simple device hich we have found convenient for the eckman B type of photometer. The water ntainer is made of black plastic, and the st tubes are supported in a shield or block 3) through which the light beam may pass a vertical slit (C). To mount the container the photometer, the standard supporting ck is removed, and the container is ad- sted into its correct position and fixed rmly in its place.

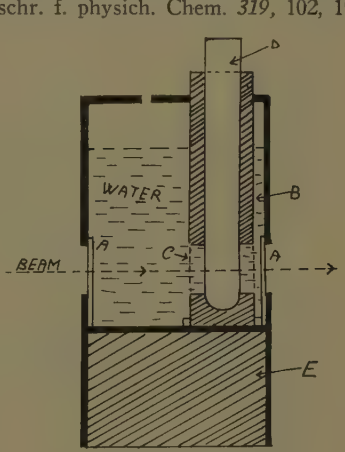
As the whole container is filled with water, e "water blank" for zero adjustment of the notometer can be omitted. Zero adjustment ntrol is automaticaly obtained whenever e samples are removed or replaced. In the lirect reading" type of photometer the only anipulation thus necessary is the transferr- g of tubes to the container and the writing wn of readings. If this is done by two diff- ent persons, more than one hundred samp- les can be measured in less than twenty minutes.

The errors due to the use of tubes rather than photometer cells of the standard type are reduced when the tubes are immersed in water, due to the more favorable refraction index. The tubes to be used must fit closely in the block and should be selected carefully from the same series. For calibration the reading of cobalt sulphate in dilute sulfuric acid at 515 mμ was used.

This device has proved to be extraordinarily time-saving when large series of samples have to be coped with.

REFERENCES

Stegemann, H.: Bestimmung von Aminosaüren mit dithionitreduzirten Ninhydrin. Hoppe Zeyler's Ztschr. f. physich. Chem. *319*, 102, 1960.



Fif. 1. The use of immersed test tubes as photometer cells. A — "windows", B — solid block, C — vertical slit, D — test tube, E — fundament or mounting.

stitute for Medical Biochemistry
d Physiology,
niversity of Oslo, Norway.
ecember 7, 1960.

I. Øye
J. Fog.

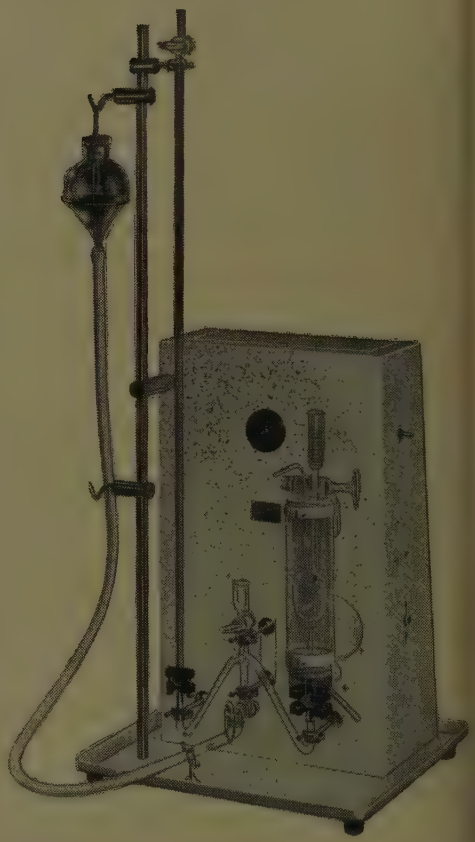
easier . . . faster . . . cleaner determinations

with our new magnetically agitated

VAN SLYKE APPARATUS

CO_2 in plasma and O_2 in whole blood can be extracted within the standard time of 3 minutes. Meanwhile the technician can load the reaction pipette funnel for the next determination. Magnetic agitation, minimising noise, vibration and risk of broken glass.

- ★ No shaking parts — no glass fatigue.
- ★ Easy quiet operation.
- ★ Trouble-free magnetic stirring.
- ★ Standard sized glassware — ready replacements.
- ★ Rapid dismantling for cleaning.
- ★ Large stopcocks accurately ground to retain vacuum — can be removed for greasing without dismantling the other glassware.
- ★ Large mercury tray — easy-clean surface.
- ★ Metal stand — rust-proofed and attractive.



Ask for details of this and the

RAPPAPORT APPARATUS

a new micro method for blood gas analysis. Only 0.1 ml sample of plasma or serum required.

Gallenkamp SUPPLY THE WORLD'S LABORATORIES

A. GALLENKAMP & CO. LTD., SUN STREET, LONDON, E.C.2 ENGLAND

APPOINTED AGENT FOR NORWAY

NERLIENS KEMISK-TEKNISKE AKTIESELSKAP

Tollbodgt. 32, Oslo

APPOINTED AGENT FOR SWEDEN

SIGURD HOLM AB

Olshammarsgt. 89, Bandhagen. Tel: 86 82 32, 86 82 42, 86 82 80

Leitz

Optiska instrument

Monokulära och binokulära mikroskop
i nyaste utförande med inbyggd belysning för genom- och påfallande ljus.

Faskontrastanordning

Storfältplanoptik

Stereomikroskop

Mikrofotografiska anordningar

Mikrotomer
för framställning av finaste mikroskopiska snitt

Fotometer

Polarimeter Refraktometer
Spektroskop

Småbildkameran LEICA
med kompletteringsanordningar för vetenskaplig och teknisk fotografi

Kataloger och prospekt på begäran

Optiska instrument
av märke **ERNST LEITZ WETZLAR**
åtnjuta inom vetenskap och teknik obestritt anseende för högsta precision och tillförlitlighet. Generationers fackkunskap och ständig vetenskaplig utveckling är även i framtiden den säkra grunden för varje LEITZ-produkt.

Generalagent sedan 1907:

AXEL LUNDQVIST AB

Drottninggatan 28 · Stockholm · Tel. 10 87 30

Egen serviceverkstad

BB KONSTRIKTIONSPIPETTER..

Hver pipette er omhyggeligt udmålt og indstiller sig automatisk på sit rumfang. Pipetterne leveres emballeret i klare plasticrør, der yder god beskyttelse under opbevaringen.

BB konstriktionspipetter leveres i størrelserne 1—3000 cmm.

BIE & BERNTSEN

PILESTRÆDE 35, KØBENHAVN K

Mettler

Analysevekter og presisjonsvekter

LEYBOLD

Vakumpumper og vakumutstyr

Radiometer elektrokjemiske måleapparater

REICHERT WIEN

Mikroskop og optisk utstyr

Representant for Norge

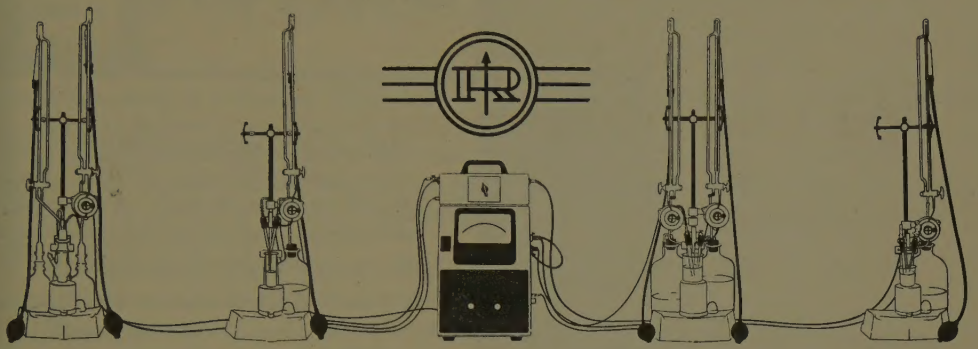
NERLIENS KEMISK-TEKNISKE AKTIESELSKAP

V. Torgv. 5, Bergen
tlf. 15 981

Tollbodgt. 32, Oslo
Sentralbord 41 73 43

Kjøbmannsgt. 34, Trondheim
tlf. 20 469

RADIOMETER AUTOMATIC TITRATOR, type TTT 1



TTA 1/KF
KARL FISCHER TITRATIONS

TTA 1
BLOOD SUGAR TITRATIONS

TTT 1/ELS 12

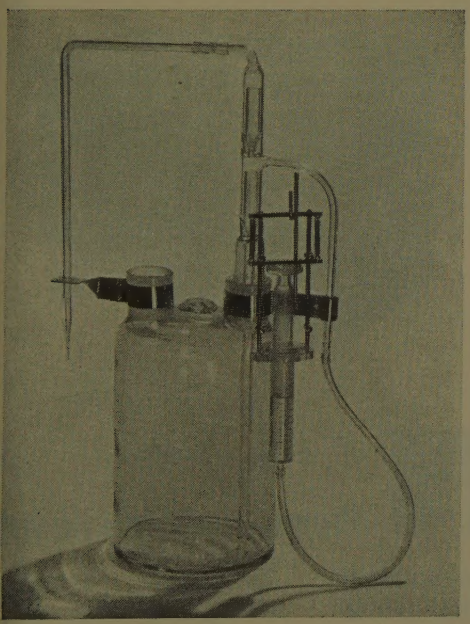
TTA1
ACID BASE TITRATIONS

TTA 1
CHLORIDE TITRATIONS

— Ideal also for all other potentiometric titrations inclusive of titrations at constant pH, e.g. determinations of choline esteraze

RADIOMETER

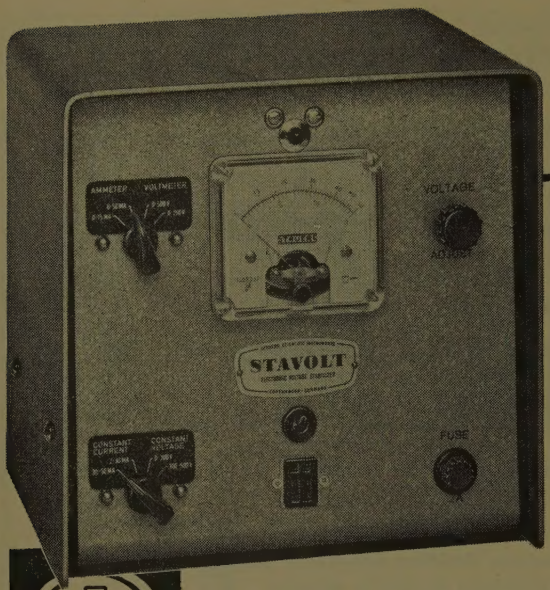
72, EMDRUPVEJ, COPENHAGEN, DENMARK



D.L. büretten

Dansk Laboratorieudstyr A/S

3, Ryesgade *Luna 2600 København N



Stavolt

Elektronisk spændingsstabilisator til papir- elektroforese. Tilsluttes 220 volt vekselstrø 50—60 perioder, og giver en vel reguleret stabiliseret spænding og/eller strøm og derv yderst reproducérbare arbejdsbetingelser.

- 4 indstillingsmuligheder: 0—250 volt, 250 500 volt, 2—30 milliampère og 30—50 mil ampère.
- Maximalvariation ± 1 % ved netspændin variationer ± 10 %.
- Udvendige mål: 260 × 200 × 215 m/m.

STRUERS

H. Struers chemiske laboratorium

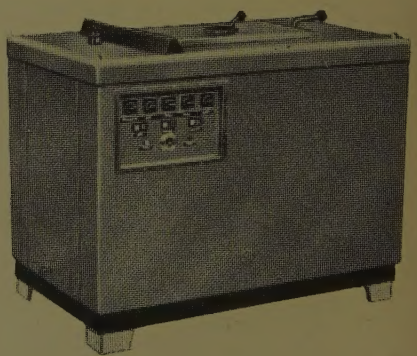
KØBENHAVN C. 1402 • AARHUS 31611 • ODENSE 12360

MARIUS DISKMASKIN FÖR LABORATORIEGLAS

— en apparat som uppfyller laboratoriernas höga krav på absolut rent laboratorieglas.

Med *Marius Diskmaskin* kan högsta grad av renhet ernås och resultatet är analogt med det som erhålles när glas enl. tidigare metod syratvättats och spolats med destillerat vatten.

För närmare upplysningar och prospekt tag kontakt med oss.



främst för laboratoriet —

RUDOLPH GRAVE AB

ETABLERAD 1874

STOCKHOLM TEL 23 37 80 | GÖTEBORG TEL 27 51 35 | MALMÖ TEL 38125

NOTICE TO AUTHORS

Manuscripts should be typewritten in English, double spaced throughout.

Tables, diagrams and other inserts should be sent on separate sheets, bearing the author's name. The legends for the figures should be typed separately, and the figures should be comprehensible without reference to the text.

All figures and illustrations are to be submitted in such a form as to permit photographic reproduction without retouching or re-drawing. They should be trimmed to the smallest area and be approximately twice the size of the finished block. Diagrams should be in Indian ink and should be drawn on plain white paper, tracing paper, Bristol board or faintly blue-lined paper. For photographs glossy prints, unmounted and not retouched, are required. Lines in line drawing, letters, numbers etc. should be of dimensions to stand reduction, usually to one-half or one-third. Unsuitable figures will be re-drawn by the press. All expenses of re-drawing, will be charged to the author.

The introduction should contain a clear statement of the purpose of the investigation and its relation to other works in the same field, but extensive reviews must be avoided.

The experimental procedure must be clearly described. However, methods should be given in extenso only if they represent a new approach.

Every paper should end with a brief summary. References to the literature should be listed alphabetically at the end of the paper, and the names of the authors should be mentioned in the text with the year of publication in brackets. The bibliography should conform to the style used in this journal: The author's name, initials, title of paper, abbreviation of journal, volume, page and year; in the case of books, the title in full, with the place and year of publication, and page.

No paper should exceed 12 printed pages. — Short reports of important findings, occupying not more than 2 printed pages, will be accepted for prompt publication in the form of *Letters to the Editors*.

CONTENTS

T. K. I. LARMI and R. APPELQVIST: Measurement of the elasticity and compliance of the lungs. Description of a method and presentation of some clinical observation 167
T. K. I. LARMI and R. APPELQVIST: The influence of cardiac surgery on the mechanical properties of the lungs 174
J. GEORG and K. MELLEMGAARD: Sources of error in the determination of metabolic rate 180
I. STÅHLE: The helium dilution method in determination of ventilatory disturbances. Its application to bronchospirometry 186
O. SIGGAARD ANDERSEN: Sampling and storing of blood for determination of acid-base status 196
O. SIGGAARD ANDERSEN: Factors affecting the liquid-junction potential in electrometric blood pH measurement 205
O. HUSOM: The particulate nature of intrinsic prothrombinase 212
O. HUSOM: Intrinsic prothrombinase: Investigations on the possibility of its formation by successive coating of intermediates on activated surfaces 216
B. ZAAR and A. GRÖNWALL: A micro method for determination of barbiturates in serum as mercuric complexes 225
AA. LADEHOFF and J. RASMUSSEN: Fibrinolysis and thromboplastic activity in relation to hemorrhage in transvesical prostatectomy 231
H. BERLIN, R. BERLIN, G. BRANTE and S.-G. SJÖBERG: Studies on intrinsic factor and pernicious anemia. III Intrinsic factor activity and B_{12}-binding power of electrophoretic fractions of intrinsic factor concentrates 245
M. ANDERSEN: The effect of different pathological conditions on the excretion of urea in the rabbit kidney 249
P. FYLLING: Serum lactic dehydrogenase activity in umbilical cord, at the end of labor in normal women, and the uncomplicated puerperium 264
F. KIIL and K. AUKLAND: Effect og high ureteral pressure on urine concentration during water diuresis 268
F. KIIL and K. AUKLAND: Renal concentration mechanism and hemodynamics at increased ureteral pressure during osmotic and saline diuresis 276
K. PIND and A. FAURBYE: The excretion of vanilmandelic acid in the urine of schizophrenics 288
C. E. G. LUNDGREN: Oxygen dissociation curve of human blood equilibrated with high concentrations of oxygen 291
E. ASMUSSEN and M. NIELSEN: The disappearance of oxygen from shed blood at body temperature 297
I. HOLM-JENSEN: Rapid determination of urea by a modified Conway technique 301
H. C. GODAL: The influence of heparin on the fibrin clot 306
H. C. GODAL: Heparin tolerance and the plasma proteins 314
A.-C. TEGER-NILSSON: Serum creatinine determination using an ion exchange resin 326
K. B. BJÖRNESJÖ: Simultaneous determination of cycloserine and pyrazinamide in blood serum 332
A. W. S. SØRENSEN: Phenacetin consumption in relation to 24-hour endogenous creatinine clearance 337

Reviews:

E. J. KING: History of the international federation of clinical chemistry 341
M. E. FREEMAN: Clinical enzyme unitage 346

Technique and Practical Problems:

I. HOLM-JENSEN: A simple mechanical stirring device. Its application to rapid determination of carbonates by a modified Conway technique 347
O. SIGGAARD ANDERSEN and K. JØRGENSEN: A gasometric apparatus for direct reading determination of carbon dioxide concentration in gas mixtures 349
K. JØRGENSEN: Rectangular cuvettes as self-emptying cells 351

Letters to the Editors:

J. BERGSTRÖM, J. FINDOR and E. HULTMAN: The contents of glycogen and potassium in human liver tissue obtained by needle biopsy 353
I. ØYE and J. FOG: The use of immersed test tubes as photometer cells 355

DISTRIBUTED BY EJNAR MUNKSGAARD, COPENHAGEN, DENMARK

PRINTING ARRANGEMENTS BY OSLO UNIVERSITY PRESS, OSLO, NORWAY

PRINTED BY A. W. BRØGGERS BOKTRYKKERI A/S, OSLO